Remarks

Reconsideration of this Application is respectfully requested.

Claims 1, 2, 37, 38 and 53 are pending in the application, with claims 1, 37 and 53 being the independent claims. Claims 1, 2, 37, 38 and 53 are sought to be amended. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

I. Support for Amendments

A. In the Title

The title has been amended in accordance with the Examiner's suggestion. *See* Paper No. 21, page 3. Support for the amendment can be found throughout the specification, for example, at page 12, lines 5-15 and in claim 1 as originally filed.

B. In the Specification

1. Cross-Reference to Related Applications

A paragraph captioned "Cross Reference to Related Applications" has been added to the specification to indicate that this applications is a 371 of PCT/US98/04683, filed

March 11, 1998 and published under PCT Article 21(2) in English on September 17, 1998, which claims the benefit of the filing date of U.S. Patent Application No. 08/816,122, filed March 11, 1997, now abandoned. Support for this paragraph can be found, for example, in the Original Declaration executed by the inventors and submitted to the USPTO on June 6, 2000.

2. Description of the Figures

The paragraph on page 19, lines 3-5, has been amended to include reference to the appropriate sequence identifier. This change therefore does not introduce any new matter.

The paragraph on page 21, lines 21-24, has been amended to more clearly describe the results depicted in Figures 19A-19E. Support for these changes can be found, for example, in the specification at page 93, line 23 through page 94, line 6, and in Figures 19A-19E as originally filed.

The paragraph on page 22, lines 19-25, has been amended to specify that the data in the "left panels" corresponds to Figure 26A and that the data in the "right panels" corresponds to Figure 26B. Support for these changes can be found, for example, in the specification at page 105, lines 16-27, and in Figures 26A and 26B as originally filed.

The paragraph on page 23, lines 13-28 has been amended to indicate that the data in Figure 30A is divided into Figures 30A-1 and 30A-2. Support for this change can be found in Figures 30A-1 and 30A-2 as originally filed.

3. Sequence Listing

In accordance with 37 C.F.R. § 1.821(c), a paper copy of a Sequence Listing is being submitted herewith for insertion at the end of the specification. The sequence listing includes a listing of the amino acid sequence depicted in Figure 6. The Sequence Listing therefore is supported by Figure 6 as originally filed and does not include any new matter. *See* 37 C.F.R. § 1.821(g).

Also submitted herewith is a copy of the Sequence Listing in computer readable form. *See* 37 C.F.R. § 1.821(e). In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Sequence Listing and the computer readable copy of the Sequence Listing submitted herewith in the above application are the same.

C. In the Claims

Support for the amendments to claims 1, 2, 37, 38 and 53 can be found throughout the specification, for example, at page 12, lines 5-15, page 16, lines 7-14, page 17, lines 10-14, and in claims 1, 2, 37, 38 and 53 as originally filed.

II. Sequence Compliance

According to the Office Action, the application does not comply with the requirements of 37 C.F.R. §§ 1.821-1.825 because the amino acid sequence in Figure 6 is not accompanied by a reference to the relevant sequence identifier. *See* Paper No. 21, page 2.

Applicants submit herewith a Sequence Listing for the amino acid sequence depicted in Figure 6. The sequence of Figure 6 has been designated SEQ ID NO:1. In accordance with MPEP § 2422.02, the brief description of Figure 6 has been amended to identify the sequence referred to therein as SEQ ID NO:1. Accordingly, the application is now in full compliance with the requirements of 37 C.F.R. §§ 1.821-1.825.

III. Information Disclosure Statement

According to the Office Action, the information disclosure statements (IDSs) filed on August 14, 2000, November 30, 2000, December 27, 2000, January 16, 2001, and February 15, 2001 fail to comply with 37 C.F.R. § 1.98(a)(2). *See* Paper No. 21, pages 2-3. Apparently, it is asserted that legible copies of the documents cited in the aforementioned IDSs were not submitted with the IDSs when filed. It was also asserted that "*there is no information disclosure statement present in the application prior to the statement of 14 August 2000.*" Paper No. 21, page 3 (emphasis in original).

Applicants note that legible copies of all the documents that were cited in the IDSs of August 14, 2000, November 30, 2000, December 27, 2000, January 16, 2001, and February 15, 2001 were submitted to the USPTO along with the corresponding IDS pleadings and PTO-1449 forms. Enclosed herewith are photocopies of the return postcards, date-stamped by the USPTO OIPE, acknowledging receipt of the documents that are cited in these IDSs.

Applicants also note that two IDSs were submitted in this Application prior to August 14, 2000: The first was submitted on January 18, 2000, and the second was

submitted on March 14, 2000. Enclosed herewith are photocopies of the return postcards, date-stamped by the USPTO OIPE, acknowledging receipt of the IDS pleadings, PTO-1449 forms and documents listed thereon, that were filed on January 18, 2000 and March 14, 2000.

Applicants respectfully submit that the requirements of 37 C.F.R. § 1.98(a)(2) have been met with regard to the IDSs that have been submitted in this application. Applicants therefore request that the information that was cited and submitted to the USPTO with the IDSs of January 18, 2000, March 14, 2000, August 14, 2000, November 30, 2000, December 27, 2000, January 16, 2001, and February 15, 2001, be considered by the Examiner.

IV. Objections to the Specification

The disclosure was objected to for the following alleged informalities:

A. Reference to Prior Applications

It is noted in the Office Action that an application which claims the benefits of an earlier application must contain a specific reference to the prior applications in the first sentence of the specification. *See* Paper No. 21, page 3.

Applicants have added to the specification a paragraph captioned "Cross Reference to Related Applications." This paragraph provides specific reference to the prior applications, the benefits of which are claimed in the present application. Thus, the requirements of 37 C.F.R. § 1.78(a)(2) and (a)(5) are fully satisfied.

B. Brief Description of the Drawings

It was noted in the Office Action that the description provided for Figures 19A, 19B and 19C "doesn't seem to match the Figures." Paper No. 21, page 3. It was also noted that the *Brief Description of the Figures* does not refer to Figures 19D, 19E, 19F, 26A, 26B, 30A-1 and 30A-2. *See* Paper No. 21, page 3.

The apparent inconsistencies in the descriptions for Figures 19A, 19B and 19C, and the absence of specific references to Figures 19D, 19E, 19F, 26A, 26B, 30A-1 and 30A-2 reflect inadvertent typographical errors. The descriptions for these figures have been amended to correct these errors.

C. Title of the Invention

It was asserted in the Office Action that the title of the invention is not descriptive and that "a new title is required that is clearly indicative of the invention to which the claims are directed." Paper No. 21, page 3.

In order to expedite prosecution, Applicants have amended the title of the invention in accordance with the Examiner's suggestion.

V. Objection to the Claims

Claims 1, 2, 37, 38 and 53 were objected to for reciting non-elected species. *See* Paper No. 21, pages 3-4. Claims 1, 2, 37, 38 and 53 have been amended to recite only the elected species. These amendments are made without prejudice to or disclaimer of the non-elected species. Applicants reserve the right to prosecute claims directed to the non-elected

species in one or more divisional applications. The objection to the claims has been fully accommodated and therefore should be withdrawn.

VI. Claim Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1, 2, 37 and 38 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. *See* Paper No. 21, page 4. Applicants respectfully traverse this rejection.

Three general bases for this rejection have been presented in the Office Action. The first basis relates to the absence in the specification of a working example that demonstrates the treatment of amyloidosis in a subject. Applicants respectfully submit that the absence of a working example demonstrating the treatment of amyloidosis in a subject does not support the rejection under 35 U.S.C. § 112, first paragraph.

The present inventors have demonstrated that metal chelators, including bathocuproine, promote the solubilization of Aβ from human brain homogenates. *See* specification at page 91, line 18 through page 94, line 25. The inventors also discovered the relationship that exists between dose of chelator used and the extent to which Aβ is resolubilized. *See* specification at page 94, lines 7-25. With regard to bathocuproine in particular, it was discovered that there is a clear dose-dependent increase in Aβ extraction from human brain. *See id.* at page 94, lines 22-25 and Figure 19E. The inventors also found that, as with human brain, homogenates of brain cortical tissue prepared from an amyloid-

bearing APP transgenic mouse in the presence of a chelator exhibit enhanced extraction of pelletable Aβ. *See id.* at page 95, lines 16-18.

The Examiner has acknowledged the findings presented in the specification, *see* Paper No. 21, pages 4-5, but nonetheless asserted that "the specification of the instant application does not teach treating amyloidosis in a subject. The specification does not teach any methods or working examples that indicate administration of bathocuproine or indomethacin/indomethacin [sic: bathocuproine/indomethacin] to a subject." Paper No. 21, page 5. With respect to claims 37 and 38, directed to pharmaceutical compositions, the Examiner stated: "the specification does not teach how to use a bathocuproine or indomethacin/indomethacin [sic: bathocuproine/indomethacin] 'pharmaceutical' composition without undue experimentation for the treatment of a disease *in an animal*." Paper No. 21, page 6 (emphasis in original).

At the outset, Applicants respectfully note that the absence of a working example is not sufficient to establish a *prima facie* case of non-enablement. *See Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ2d 1302, 1304 (Fed. Cir. 1987). The specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art would have been able to practice it without undue experimentation. *See In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970). As discussed in more detail below, the specification, supplemented with the knowledge possessed by those of ordinary skill in the art, would have provided sufficient guidance for practicing and/or making and using the subject matter encompassed by the present claims. Thus, the absence

of a working example in the specification does not support the rejection for lack of enablement.

Moreover, in order to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office . . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971) (emphasis in original).

In the present Office Action, there has been no evidence or explanation set forth to explain why the *in vitro* results presented in the specification are not indicative of *in vivo* results. That is, there has been no evidence presented to suggest that the ability of bathocuproine to promote the solubilization of Aβ *in vitro* would not reflect the ability of bathocuproine to promote the solubilization of Aβ when administered to a subject suffering from amyloidosis.

In fact, the existing evidence and teachings in the art strongly support the correlation between the *in vitro* results presented in the specification and the results that would be obtained a subject.

First, the physiological conditions that exist in brain homogenates (*e.g.*, pH, ion concentration, macromolecular content, etc.) closely approximate the conditions found in the brain tissue environment *in vivo*.

Second, it was known in the art at the time the application was filed that the regulation of zinc and copper in the brain is abnormal in AD and that these metals are integral components of the Aβ deposits in the brains of AD patients. *See* specification at page 90, line 27 through page 91, line 4. It was also observed that zinc- and copper-specific chelators dramatically redissolve a significant proportion of Aβ extracted from post-mortem AD affected brain tissue. *See* specification at page 91, lines 4-8.

Third, a particular strategy described in the art (albeit an impractical one) that resulted in slowing the progression of AD involved the intramuscular administration of desferrioxamine, a chelator of copper and zinc, to AD patients. *See* Crapper-McLachlan *et al.*, *Lancet 337*:1304-1308 (1991).

In view of the foregoing, it is reasonable to conclude that the *in vitro* results obtained with bathocuproine (and the other chelators described in the specification) are indicative of the results that would be achieved when the chelator is administered to a subject; *i.e.*, it is reasonable to conclude that the administration of bathocuproine to a subject would result in the treatment of amyloidosis. There has been no specific evidence presented to contradict the logic underlying this conclusion.

In support of the assertion that the results obtained in the *in vitro* system described in the specification accurately reflect the results that would be obtained in a subject, Applicants point to the analogous circumstances involving the chelator clioquinol. Clioquinol, like bathocuproine, is a metal chelator that was first shown to promote the solubilization of Aβ *in vitro*. *See* Cherny *et al.*, *Neuron 30*:665-676 (2001) (copy attached hereto as Exhibit 1). Based on the *in vitro* results with this chelator, clioquinol was

subsequently administered to a transgenic mouse model of AD and was found to effectively inhibit brain Aβ deposition. *See id.* More recently, clioquinol was shown to improve cognitive parameters and blood levels of Aβ when administered to humans in a clinical trial. *See* Ritchie *et al.*, "Metal complexation with iodochlorhydroxyquin (clioquinol) targeting Aβ amyloid deposition and toxicity in Alzheimer's disease: proof-of-concept and safety" (unpublished manuscript, submitted for publication) (2003) (copy attached hereto as Exhibit 2).

Thus, the *in vitro* results involving the chelator clioquinol accurately reflected the results obtained *in vivo*, *i.e.*, the treatment of amyloidosis in subjects. The results with clioquinol indicate that a similar progression (*i.e.*, from *in vitro* data to *in vivo* results) in the context of bathocuproine could have likewise been achieved using only routine experimentation.

The second basis for the rejection is the assertion that "[u]ndue experimentation would be required of the skilled artisan to determine the optimal quantity of bathocuproine or bathocuproine/indomethacin to be administered to a subject as well as the optimal duration of treatment and route of administration." Paper No. 21, page 5. Applicants respectfully disagree with this assessment.

The evidence presented above for clioquinol demonstrates that the process of determining dosage amount, duration of treatment and route of administration for a chelator, in the context of treating amyloidosis, can be determined using only routine experimentation. In addition, the specification provides substantial guidance for determining the quantity of chelator to be administered as well as the timing and acceptable routes of administration.

For instance, the specification notes that, when administering chelators to a patient, it is important to vary the dosages of chelator "so that during the course of administration, chelator concentrations will be varied frequently to randomly allow achieving the most effective concentration for dissolving Aβ amyloid deposits in the given patient." Specification at page 45, lines 7-9. With respect to bathocuproine in particular, the specification teaches that there is a dose-dependent increase in Aβ extraction from human brain in the presence of this chelator. *See* specification at page 94, lines 22-25; *see also* page 95, Table 2.

The specification also describes considerations that influence the timing of administration of the chelators depending on factors such as the degree to which an individual is affected with amyloidosis. *See* specification at page 45, lines 10-27.

With respect to routes of administration, the specification states that "the active agents may be administered in any convenient manner either orally or parenteraly . . ." Specification at page 46, lines 8-9. The specification further describes various considerations that should be taken into account in determining the route of administration such as, *e.g.*, the ability of the agents to cross the blood-brain barrier, the solvents that can be used in injectable solutions, the degree to which active ingredients are protected to permit oral administration, the inclusion of other ingredients, etc. *See id.* at page 46, line 8 through page 49, line 3.

Thus, the specification would have provided sufficient guidance as to the amount of bathocuproine that should be administered to a subject, the timing of administration and the possible routes of administration.

Moreover, an Applicant is not limited to the confines of the specification to provide the necessary information to enable an invention. *See In re Howarth*, 654 F.2d 103, 105-6, 210 USPQ 689, 692 (CCPA 1981). An Applicant need not supply information that is well known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997); *Howarth*, 654 F.2d at 105-6, 210 USPQ at 692; *see also In re Brebner*, 455 F.2d 1402, 173 USPQ 169 (CCPA 1972). Persons of ordinary skill in the art would have known, generally, how to determine the amount of an active ingredient to include in a pharmaceutical composition, the appropriate timing of administration and the appropriate routes of administration. Determining such factors was a matter of routine optimization in the art as of the effective filing date of the application. Therefore, a person of ordinary skill in the art, in view of the specification, would have been able to practice and/or make and use the subject matter of the claims with only routine experimentation.

The third basis for the rejection under 35 U.S.C. § 112, first paragraph, is the assertion that "[o]ne skilled in the art would also not be able to predict the effects of bathocuproine or indomethacin/indomethacin [sic: bathocuproine/indomethacin] might have in a subject . . ." Paper No. 21, page 5. To support this assertion, the Office Action cites Gillmore *et al.*, *Brit. J. Haematol. 99*:245-256 (1997). The passage cited in Gillmore (*i.e.*, the paragraph bridging pages 249-250), however, merely indicates that, at the time of this reference, research in the area of amyloid deposit mobilization was *ongoing*. The fact that others in the art had not been able to accomplish the results provided by the present invention cannot form the basis for a proper enablement rejection. *See Gould*, 822 F.2d at 1078, 3 USPQ2d at 1304.

Furthermore, there is no discussion whatsoever in Gillmore relating to the use of metal chelators in treating amyloidosis. Gillmore, therefore, provides no basis for assessing the predictability of the effects of a chelator on a subject. There is nothing in Gillmore to suggest that the effects of bathocuproine or bathocuproine/ indomethacin in a subject would have been regarded as unpredictable.

In summary, the present specification clearly demonstrates the ability of bathocuproine (and other chelators) to promote the solubilization of Aβ in brain sample homogenates. It is appreciated in the art that *in vitro* results generally reflect the results that would be obtained *in vivo*, especially when the *in vitro* experiments closely approximate the conditions that are found *in vivo*, as is the case with the experiments in the present specification. There has been no evidence presented to suggest that the experimental results presented in the specification for bathocuproine would not also be obtained when the chelator is administered to an animal. As of the effective filing date of the application, persons of ordinary skill in the art routinely determined the appropriate quantity, timing and route(s) of administration of compounds in pharmaceutical formulations. Finally, persons of ordinary skill in the art were able to proceed experimentally from *in vitro* results with the metal chelator clioquinol to the effective treatment of amyloidosis in subjects (including a successful clinical trial with humans), using routine experimentation and methods that were available as of the effective filing date of the present application. The clioquinol example indicates that similar successes would likely be achieved with bathocuproine and the other chelators described in the specification.

Thus, a person of ordinary skill in the art, in view of the present specification, would have been able to practice and/or make and use the subject matter encompassed by the present claims using only routine experimentation. Accordingly, Applicants respectfully request that the rejection of claims 1, 2, 37 and 38 under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

VII. Claim Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 1, 2, 37, 38 and 53 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. *See* Paper No. 21, page 7. Two separate bases for this rejection were set forth in the Office Action, both of which are addressed in turn as follows.

A. Acronyms in Claims 1, 2, 37, 38 and 53

The Examiner stated that the acronyms "Abeta," "TETA," and "TPEN" in claims 1, 2, 37, 38 and 53, render these claims indefinite. *See* Paper No. 21, page 7.

Claims 1 and 37 have been amended to replace "Abeta" and "Aβ," respectively, with "amyloid beta." The abbreviations "TETA" and "TPEN" are no longer found in the claims as currently presented. Thus, this basis of rejection has been fully accommodated and should be withdrawn.

B. A Step that Clearly Relates Back to the Preamble in Claims 1 and 2

The Examiner stated that "[c]laims 1-2 are indefinite because the claims do not have a step that clearly relates back to the preamble." Paper No. 21, page 7. Applicants respectfully traverse this basis of rejection.

Claim 1, in its present form, recites:

> A method of treating amyloidosis in a subject, said method comprising administering to said subject an effective amount of (a) bathocuproine or a hydrophobic derivative thereof; and (b) one or more pharmaceutically acceptable carriers or diluents; *for a time and under conditions to bring about said treatment*; and . . .
> (Emphasis added).

The expression "for a time and under conditions to bring about said treatment" clearly relates back to the preamble ("a method of treating amyloidosis in a subject.") Thus, claims 1 and 2 are not indefinite. Accordingly, Applicants respectfully request that the second basis for the rejection of claims 1 and 2 under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.

VIII. Claim Rejections Under 35 U.S.C. § 103

Claim 37 was rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over the Sigma Chemical Company catalog number B 1000, page 149 (1995) (hereinafter "Sigma") in view of Goodman and Gilman, "The Pharmacological Basis of Therapeutics," New York: McGraw-Hill, Inc., pages 5-6 (1993) (hereinafter "Goodman"). *See* Paper No. 21, page 7. Applicants respectfully traverse this rejection.

In order to establish a *prima facie* case of obviousness, there must be some suggestion or motivation to modify the references or to combine reference teachings. *See In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998). The evidence demonstrating a motivation to combine references must be "clear and particular." *See In re Dembiczak*, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999). "Broad conclusory statements regarding the teaching of multiple references, standing alone, are not 'evidence.'" *Id.*, 175 F.3d at 999, 50 USPQ2d at 1617.

Claim 37, in its current form, is directed to a pharmaceutical composition for the treatment of conditions caused by amyloidosis, Aβ-mediated ROS formation, or both, comprising: (a) bathocuproine or a hydrophobic derivative thereof; and (b) one or more pharmaceutically acceptable carriers or diluents.

Sigma simply lists bathocuproine as one of many chemical compounds. Sigma does not provide any indication or suggestion that bathocuproine can be included in a pharmaceutical composition or that it can be combined with a pharmaceutically acceptable carrier or diluent.

Goodman provides only a general statement regarding factors that influence the absorption of drugs. *See* Goodman at page 5, bottom right column ("Drugs given in aqueous solution are more rapidly absorbed than those given in oily solution, suspension or solid form. . .") There is nothing in the cited passage from Goodman, however, that suggests combining a pharmaceutically acceptable carrier or diluent with bathocuproine or any other metal chelator. In fact, Goodman does not mention metal chelators at all.

Thus, neither Sigma nor Goodman provide any specific suggestion or motivation to combine bathocuproine or a hydrophobic derivative thereof with one or more pharmaceutically acceptable carriers or diluents. The legal requirement for "clear and particular" evidence of a motivation to combine reference teachings has not been met. *See Dembiczak*, 175 F.3d at 999, 50 USPQ2d at 1617.

It is asserted in the Office Action that "[t]he person of ordinary skill in the art would have been motivated to make that modification [*i.e.*, combining a metal chelator with an aqueous diluent or carrier] because drugs put into an aqueous solution are more rapidly absorbed in a subject." Paper No. 21, page 8. Applicants respectfully submit that this asserted justification for the rejection under § 103 is legally insufficient. Simply because drugs *in general* are more rapidly absorbed in a subject when put into an aqueous solution, does not in any way suggest the *specific* combination of a metal chelator with an aqueous solution.

It is important to note that the mere fact that an advantage might be realized by combining reference teachings does not mean that a skilled artisan would be motivated to make the combination. *See In re Mills*, 916 F.2d 680,682, 16 U.S.P.Q.2d 1430, 1432 (Fed. Cir. 1992) (Although a prior art device "may be capable of being modified to run the way the apparatus is claimed, there must be a suggestion or motivation in the reference to do so.") *See also In re Gordon*, 733 F.2d 900, 902, 221 USPQ 1125, 1127 (Fed. Cir. 1984) ("The mere fact that the prior art could be so modified would not have made the modification obvious unless the prior art suggested the desirability of the modification.") Thus, the fact that bathocuproine *could have been* combined with an aqueous solution does

not constitute a legally sufficient motivation for one of ordinary skill in the art to make the combination.

In addition, there has been nothing specifically cited that indicates the need or desirability of improving the absorption of metal chelators in a subject. Thus, the assertion that "drugs put into an aqueous solution are more rapidly absorbed in a subject" does not logically lead to the conclusion that a person of ordinary skill in the art would have been motivated to combine a metal chelator with an aqueous solution.

Since there has not been any specific and particular evidence put forth to indicate a motivation to combine bathocuproine or a hydrophobic derivative thereof with one or more pharmaceutically acceptable carriers or diluents, a *prima facie* case of obviousness has not been established. Therefore, Applicants respectfully request that the rejection of claim 37 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite

prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

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Version with markings to show changes made

In the Title:

Please substitute the following Title of the Invention for the pending Title of the Invention:

A COMPOSITION COMPRISING A METAL CHELATOR AND A METHOD OF TREATING AMYLOIDOSIS BY ADMINISTERING THE METAL CHELATOR

In the Specification:

On page 1, immediately after the title, please insert the following caption and paragraph:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a 371 of PCT/US98/04683, filed March 11, 1998 and published under PCT Article 21(2) in English on September 17, 1998, which claims the benefit of the filing date of U.S. Patent Application No. 08/816,122, filed March 11, 1997, now abandoned.

On page 19, please replace the paragraph that beings on line 3 and ends on line 5 with the following:

Figure 6 shows the amino acid sequence of $APP_{669-716}$ near $A\beta_{1-42}$ (SEQ ID NO:1). Rat Aβ is mutated (R5G, Y10F, H13R; bold). Possible metal-binding residues are underlined.

On page 21, please replace the paragraph that begins on line 21 and ends on line 24 with the following:

Figures 19A[-19C shows dilution curves for TPEN, EGTA, and bathocuproine, respectively, used in extracting a representative AD brain sample.], 19C and 19E show the results of Western blot analysis of 6 AD brain samples homogenized in the presence of chelators as indicated.

Figures 19B and 19D show the results of densitometry analysis of the Western blots of Figs. 19A and 19C, respectively. Figures 19A-19[C]E show that metal chelators promote the solubilization of Aβ from human brain sample homogenates.

On page 22, please replace the paragraph that begins on line 19 and ends on line 25 with the following:

Figures 26A and 26B show[s] that chelation promotes the solubilization of $A\beta_{1-40}$ and $A\beta_{1-42}$ from AD and non-AD tissue. Representative AD ([left panels] Fig. 26A) and aged-matched control specimens ([right panels] Fig. 26B) were prepared as described in PBS or 5 mM BC. Identical gels were run and Western blots were probed with mAbs WO2 (raised against residues 5-16, recognizes $A\beta_{1-40}$ and $A\beta_{1-42}$) G210 (raised against residues 35-40, recognizes $A\beta_{1-40}$) or G211 (raised against residues 35-42, recognizes $A\beta_{1-42}$) (See Ida, N. *et al.*, *J. Biol. Chem.* *271*:22908 1996).

On page 23, please replace the paragraph that begins on line 13 and ends on line 28 with the following:

Figures 30A-30E show dissolution of SDS-resistant Aβ polymers. Figures 30A-1 and 30A-2 show[s] that chaotrophic agents are unable to disrupt polymerization. Figure 30B shows that metal ion chelators disrupt SDS-resistant $A\beta_{1-40}$ polymers. Figure 30C shows that metal ion chelators disrupt SDS-resistant $A\beta_{1-42}$ polymers. The chelators, their log stability constant, and their molecular weight, respectively, are as follows: TETA (tetraethylenediamine), 20.4, 146; EDTA (ethylenediaminetetra acetic acid), 18.1, 292; DTPA (diethylenetriaminopenta acetic acid), 21.1, 393; CDTA (*trans*-1,2-diaminocyclohexanetetra acetic acid), 22.0, 346; and NTA (nitrilotriacetic acid), 13.1, 191. Figure 30D shows that α-helical promoting solvents and low pH disrupt polymers. Aliquots of $A\beta_{1-42}$ were incubated at

pH 1 or with DMSO/HFIP (75%:25%) for 2 h (30 min., 37°C). Figure 30E shows that metal ion chelators disrupt SDS-resistant Aβ polymers extracted from AD brains. Aliquots of SDS-resistant Aβ polymers extracted from AD brains were incubated with no chelator, TETA (1 mM or 5 mM) or BC (1 mM or 5 mM) for 2 h (30 min., 37°C) and aliquots collected for analysis. Monomer $A\beta_{1-40}$ is indicated.

At the end of the specification, please insert the Sequence Listing that is appended hereto.

In the Claims:

Please substitute the following claim 1 for the pending claim 1:

1. (Twice amended) A method of treating amyloidosis in a subject, said method comprising administering to said subject an effective amount of (a) [a metal chelator selected from the group consisting of:] bathocuproine[, bathophenanthroline, penacillamine, tetraethylenediamine (TETA), N,N,N',N'-tetrakis[2-pyridyl-methyl]ethylenediamine (TPEN)] or a hydrophobic derivative[s] thereof; and (b) one or more pharmaceutically acceptable carriers or diluents; for a time and under conditions to bring about said treatment; and

wherein said chelator reduces, inhibits or otherwise interferes with [Abeta] amyloid beta peptide (Aβ)-mediated production of radical oxygen species.

Please substitute the following claim 2 for the pending claim 2:

2. (Once amended) The method of claim 1 further comprising administering to the subject an effective amount of [a compound selected from the group consisting of: rifampicin, disulfiram, and] indomethacin, or a pharmaceutically acceptable salt thereof.

Please substitute the following claim 37 for the pending claim 37:

37. (Twice amended) A pharmaceutical composition for treatment of conditions caused by amyloidosis, [Aβ] amyloid beta peptide (Aβ)-mediated reactive oxygen species (ROS) formation, or both, comprising: (a) [a metal chelator selected from the group consisting of:] bathocuproine[, bathophenanthroline, penacillamine, TETA, and TPEN,] or a hydrophobic derivative[s] thereof; and (b) one or more pharmaceutically acceptable carriers or diluents.

Please substitute the following claim 38 for the pending claim 38:

38. (Once amended) The pharmaceutical composition of claim 37 further comprising [a compound selected from the group consisting of: rifampicin, disulfiram, and] indomethacin, or a pharmaceutically acceptable salt thereof.

Please substitute the following claim 53 for the pending claim 53:

53. (Once amended) A composition of matter comprising: (a) [a metal chelator selected from the group consisting of:] bathocuproine[, bathophenanthroline, penacillamine, TETA, and TPEN,] or a hydrophobic derivative[s] thereof; and (b) [a compound selected from the group consisting of: rifampicin, disulfiram, and] indomethacin.

EXHIBIT 1

Neuron, Vol. 30, 665–676, June, 2001, Copyright ©2001 by Cell Press

Treatment with a Copper-Zinc Chelator Markedly and Rapidly Inhibits β-Amyloid Accumulation in Alzheimer's Disease Transgenic Mice

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Summary

Inhibition of neocortical β-amyloid (Aβ) accumulation may be essential in an effective therapeutic intervention for Alzheimer's disease (AD). Cu and Zn are enriched in Aβ deposits in AD, which are solubilized by Cu/Zn-selective chelators in vitro. Here we report a 49% decrease in brain Aβ deposition (−375 μg/g wet weight, p = 0.0001) in a blind study of APP2576 transgenic mice treated orally for 9 weeks with clioquinol, an antibiotic and bioavailable Cu/Zn chelator. This was accompanied by a modest increase in soluble Aβ (1.45% of total cerebral Aβ); APP, synaptophysin, and GFAP levels were unaffected. General health and body weight parameters were significantly more stable in the treated animals. These results support targeting the interactions of Cu and Zn with Aβ as a novel therapy for the prevention and treatment of AD.

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Introduction

β-amyloid peptide (Aβ), which accumulates in the neocortex in Alzheimer's disease (AD), possesses selective high- and low-affinity Cu^{2+} and Zn^{2+} binding sites that mediate both its protease resistance, reversible precipitation (Atwood et al., 1998, 2000; Bush et al., 1994a, 1994b; Huang et al., 1997), as well as the O_2-dependent production of H_2O_2 (Aβ42 > Aβ40) and concomitant toxicity (Cuajungco et al., 2000; Huang et al., 1999a, 1999b). Cu and Zn are elevated in the neocortex in AD and particularly concentrated in amyloid plaques (Lovell et al., 1998; Suh et al., 2000). We recently reported that Cu/Zn chelators solubilize Aβ from postmortem AD brain tissue (Cherny et al., 1999). Recent studies of amyloid deposits in the APP2576 transgenic (Tg) mouse model of AD (Hsiao et al., 1996) have identified enrichments of Zn (Lee et al., 1999) and Fe (Smith et al., 1997), resembling those seen in AD amyloid (Cu levels have not yet been studied in this model). Therefore, we sought to determine whether treatment with a bioavailable chelator would inhibit brain β-amyloid deposition in this Tg mouse model.

In order to identify agents for testing, we first considered existing US Pharmacopoeia (USP) drugs with established toxicology profiles, so that the initiation of clinical trials could be accelerated. Chelators such as triene (TETA), penicillamine, and desferrioxamine are safely used pharmacologically for the treatment of metal overload disorders, such as Wilson's disease. However, these molecules are hydrophilic and exert their effects by systemic depletion of metals, and do not pass across the blood-brain barrier. Hence, while we have established that these common chelating compounds do reverse metal-induced Aβ aggregation and H_2O_2 production (Atwood et al., 1998; Bush et al., 1999; Huang et al., 1997, 1999a, 1999b), they are unlikely to penetrate the brain Aβ mass in AD mouse models. There are many other USP drugs that, while not being termed chelators, have chelating properties and favorable toxicity profiles. This is generally true of the quinoline and quinolone drug class. One example is clioquinol (CQ, iodochlorhydroxyquin, 5-chloro-7-iodo-8-hydroxyquinoline, MW = 305.5), a quinoline that selectively binds Zn^{2+} and Cu^{2+} with greater affinity than it binds Ca^{2+} and Mg^{2+} [K_1(Zn) = 7.0, K_1(Cu) = 8.9, K_1(Ca) = 4.9, K_1(Mg) = 5.0]. CQ is hydrophobic and freely crosses the blood-brain barrier (Padmanabhan et al., 1989). It therefore possesses some of the ideal prototypic properties for a candidate agent

that could solubilize Zn/Cu-assembled Aβ deposits in vivo and inhibit Aβ r dox ch mistry. CQ was used extensively as an oral antibi tic (Richards, 1971) befor it was withdrawn in the early 1970s due to overdose-associated neurological side effects that are now believed to be preventable with B12 supplementation (Yassin et al., 2000).

Here we report the effects of CQ (oral treatment) on aged APP2576 Tg mice with advanced Aβ deposition. CQ treatment for 9 weeks markedly inhibited cerebral Aβ deposition by 375 μg/g wet weight compared to sham-treated controls. These changes were accompanied by no adverse effects and a significant improvement in scores on a general behavior rating scale. These findings are strong support for the role of zinc and copper interaction with Aβ in the pathophysiology of AD and indicate that the CQ class of agents could have therapeutic utility in AD.

Results

We first studied CQ in filtration assay systems that we previously used to identify several chelators that inhibit and reverse Zn/Cu-induced aggregation of synthetic Aβ1-40 and Aβ1-42 peptides in vitro (Atwood et al., 1998, 2000; Bush et al., 1994a, 1994b; Huang et al., 1997; Moir et al., 1999). CQ (2 μM, in TBS, pH 7.4) was significantly more effective than EDTA (2 μM, in TBS, pH 7.4) in dissolving Aβ1-40 aggregates induced by either Zn^{2+} or Cu^{2+} (Figure 1A). Neither chelator could resolubilize Aβ1-40 aggregates induced by incubating the peptide at pH 5.5, which induces β-sheet formation (Wood et al., 1996) (Figure 1A).

Potential binding interactions of CQ with Aβ were studied by NMR spectroscopy of Aβ1-28, which possesses the metal binding sites of Aβ centered around the 3 histidine residues (Atwood et al., 1998; Bush et al., 1994a), as well as a domain (residues 17-21) that binds to peptide fibrilization inhibitors (Soto et al., 1996). When one equivalent of Cu^{2+} was added to Aβ1-28 (1 mM in aqueous solution), the metal bound to the histidine residues as evidenced by broadened NMR peaks observed in the differences between the spectra shown in Figure 1B, scans A and B. The addition of CQ to this solution restored the peaks that had been broadened in the starting NMR spectrum of Aβ (Figure 1B, scan C) due to the presence of copper. This result is consistent with CQ removing bound copper from Aβ. There were no changes to the NMR spectrum of Aβ1-28 upon addition of CQ alone, and addition of CQ to either aqueous or DMSO solutions of Aβ1-40 (0.3 mM) did not affect the NMR spectrum (data not shown), suggesting that CQ does not act as a fibril inhibitor by binding the peptide. NMR spectra of CQ alone were recorded and found not to be perturbed by the addition of Aβ1-40 (0.3 mM), confirming that CQ does not bind to the peptide.

In order to confirm that CQ, like other Cu^{2+}/Zn^{2+}-selective chelators, could chemically solubilize Aβ deposits in AD (Cherny t al., 1999), we homogenized postmortem human brain samples affected by AD in the presenc of CQ. We found that there was a concentration-dependent increas in Aβ liberated into the soluble phase, typically >200% in the presence of ≥0.4 μM CQ (Figure

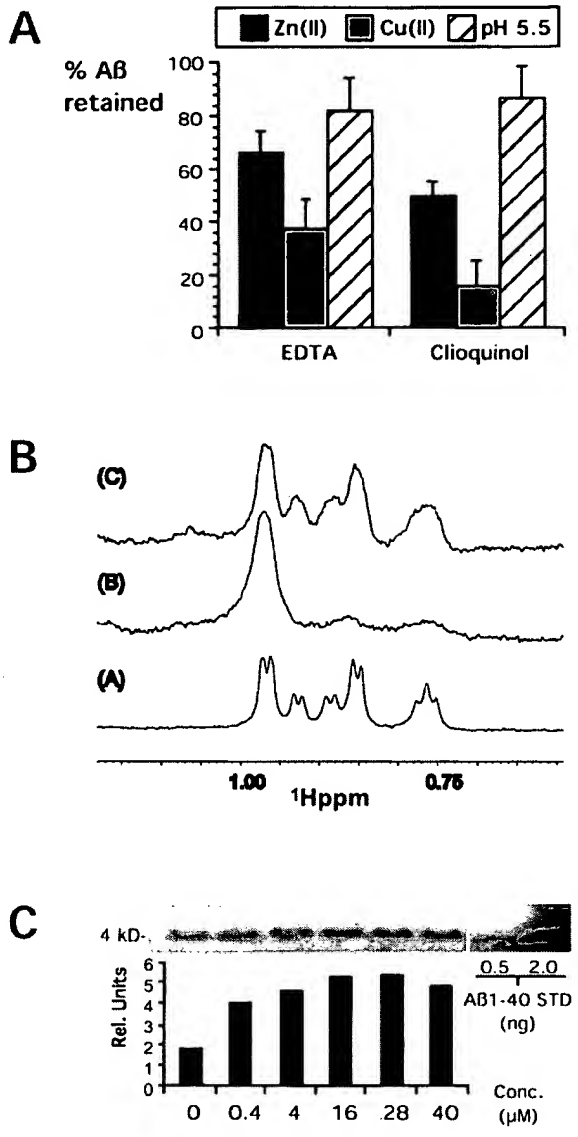

Figure 1. Interactions of Clioquinol with Aβ In Vitro

(A) Effects of CQ upon the retention of Aβ1-40 aggregates induced by Zn^{2+}, Cu^{2+}, or pH 5.5. The procedure was a modification of one which we have previously reported (Moir et al., 1999). Values are expressed as a percentage of the amount of aggregated Aβ detected after washing with TBS pH 7.4 vehicle alone (100%), represented as mean ± SD, n = 3.

(B) NMR spectroscopy of Aβ1-28 in the presence of Cu^{2+} and CQ. *A*, Spectrum of Aβ1-28 in saline buffer (pH 6.9) showing the peaks due to the methyl groups of Val-12, -18, -24 and Leu-17. *B*, Spectrum of sample in *A* after adding Cu^{2+}. *C*, Spectrum of the sample in *B* after adding CQ. The lower resolution of the spectrum in C is due to the presence of aggregated material in the sample.

(C) Enhanced extraction of Aβ from human AD-affected postmortem brain tissue following homogenization in the presence of clioquinol. The upper panel shows a Western blot (using WO2, which detects both Aβ1-40 and Aβ1-42) of the soluble fraction of frontal lobe, the same sample of which had been divided and homogenized in the presence of increasing concentrations of CQ (in PBS, pH 7.4). Corresponding densitometric quantification is shown in the lower panel. The data are representative of n = 9 AD cases.

1C, typical of 9 cases). The Aβ liberated by treatment with CQ was detected using monoclonal antibody WO2 (which detects Aβ40 and Aβ42 at an epitope between residues 5–8, Figure 1C), and a similar proportional increase in Aβ40 and Aβ42 species compared to the baseline amounts extracted by PBS was determined by blotting with G210 (specific for Aβx-40) and G211 (specific for Aβx-42) (Ida et al., 1996) (data not shown). The effect of CQ in enhancing Aβ liberation in this assay is comparable to the effect we previously reported in the same system for bathocuproine, a Cu^+ chelator (Cherny et al., 1999). Similar to the effects of homogenizing the brain sample with other Cu/Zn-selective chelators (Cherny et al., 1999), the majority (90%) of the Aβ remains in the pellet phase after one extraction with CQ. However, repeated extractions continue to liberate approximately the same proportion of Aβ so that eventually the majority of the Aβ in the tissue is solubilized (data not shown).

In view of these results, we performed a pilot study of CQ treatment in the APP2576 Tg mice (Hsiao et al., 1996). We first compared the effects of CQ and triethylene tetramine (TETA, a hydrophilic Cu/Zn-selective chelator) on a cohort of 12-month-old APP2576 mice. The drugs were delivered by gavage daily for 12 weeks. The animals were sacrificed and brain Aβ levels were appraised. There was a mean decrease in the pellet fraction of cerebral homogenates from the animals treated with CQ 2 mg/kg/d group (275 ± 38 μg/g protein, n = 6) that did not reach statistical significance compared to sham-treated controls (316 ± 81 μg/g protein, n = 6). However, there was a significant 65% decrease in the levels of sedimentable Aβ in the mice treated with CQ 20 mg/kg/d (110 ± 56 μg/g protein, mean ± SE, n = 5, $p < 0.01$). Intriguingly, two animals in the CQ 20 mg/kg/d treatment group were found to have no measurable Aβ in the brain pellet fractions and no detectable amyloid pathology in their neocortex or cortical blood vessels (Figure 2). Transgenic status and the overexpression of APP were reconfirmed in all animals. TETA at 18 mg/kg/d had no significant effect on sedimentable Aβ levels either alone (494 ± 56 μg/g protein, n = 5) or in combination with CQ 2 mg/kg/d (342 ± 114 μg/g protein, n = 5).

To further test whether the amyloid-clearing effects of CQ, we next studied the effects of CQ at a higher dose (30 mg/kg/d) in a larger cohort (n = 20 on drug, n = 19 sham-fed transgenic controls) of older (21 months) APP2576 mice using more detailed analysis. We utilized a similar blinded protocol, in which the animals were administered CQ by daily gavage for a shorter interval (9 weeks), since the animals were of advanced age. There were similar numbers of male and female mice in each treatment group after randomization. Measurement of cerebral Aβ levels at the completion of the study indicated that there was again a marked and significant decrease in pellet Aβ in CQ-treated mice. The levels of pellet Aβ in sham-treated mice were 7.48 ± 0.73 mg/g protein, 770.0 ± 68.6 μg/g wet weight, and in CQ-treated mice were 4.44 ± 0.36 mg/g protein [41% decrease, p = 0.001], 394.6 ± 39.6 μg/g wet weight [49% decrease, p = 0.0001] (Figure 3A). The difference in sedimentable Aβ of ~375 μg/g wet weight of cerebral tissue after only 9 weeks of treatment with CQ indicates a profound alteration in the rate of Aβ accumulation, which can be appreciated when contrasted to the average levels of Aβ in the pellet fraction of AD-affected cortical tissue (20 μg/g wet weight) (McLean et al., 1999) measured by the same technique.

Quantification of soluble cerebral Aβ levels in the samples revealed a significant increase in the levels of soluble Aβ in the brains of the CQ-treated mice (0.25 ± 0.02 mg/g protein [+52%, p = 0.004], 8.06 ± 0.81 μg/g wet weight [+44%, p = 0.014]) compared to sham-treated controls (0.16 ± 0.01 mg/g protein, 5.61 ± 0.41 μg/g wet weight) (Figure 3B). This rise represented a small (1%) increase in the contribution of soluble Aβ to total cerebral Aβ content (CQ-treated 2.10% ± 0.19%, sham-treated 0.81% ± 0.09%; Figure 3C). However, the increase is soluble Aβ levels is very modest (0.6 μM, assuming one g wet weight = 1 ml) compared to the profound decrease in sedimentable Aβ (approximately −90 μM) in the cerebrum in the CQ-treated mice. Soluble APP (sham, 31.9 ± 2.4 μg/g wet weight; CQ, 34.9 ± 2.5 μg/g wet weight; p = 0.41) and pellet APP (sham, 138.2 ± 19.8 μg/g wet weight; CQ, 172.8 ± 21.4 μg/g wet weight; p = 0.25) levels in these samples were not significantly different in the CQ compared to the sham-treated cohorts, indicating that the decrease in total Aβ levels induced by CQ was not due to decreased APP production.

Accompanying these changes was a significant decrease (p = 0.04) in the immunohistochemical amyloid plaque surface area in the CQ-treated mice (13.0 ± 1.5 μ^2/100 μ^2) compared to the sham-treated mice (17.3 ± 1.3 μ^2/100 μ^2) (Figure 3D). There were no correlations between the levels of soluble Aβ, total Aβ, soluble/total Aβ ratio, or plaque surface area in either the CQ-treated mice or sham-treated mice (or combined groups), which is in agreement with our previous findings of a lack of relationship between soluble Aβ, sedimentable Aβ, and plaque surface area in postmortem AD brain specimens (McLean et al., 1999).

As a marker of synaptic loss, cerebral synaptophysin levels were assayed and determined to be unaffected (sham = 788 ± 102 U/g protein, n = 14; CQ = 720 ± 63 U/g protein, n = 13, p = 0.57). There was also an insignificant 20% decrease in hippocampal cells staining positively for glial fibrillary acidic protein (GFAP) in the CQ-treated mice (sham = 27.0 ± 4.3 cells/hpf, n = 13; CQ = 21.6 ± 3.7, n = 14, cells/hpf, p = 0.34). The brain masses and protein concentrations were unchanged in the CQ-treated mice compared to the sham-treated controls (sham = 0.262 ± 0.015 g wet weight/hemisphere, 23.42 ± 1.08 mg protein/hemisphere, n = 14; CQ = 0.279 ± 0.005 g wet weight/hemisphere, 20.95 ± 1.17 mg protein/hemisphere, n = 14, p > 0.1). No significant correlations were observed between synaptophysin or GFAP levels and levels of Aβ, plaque, or total protein.

Serum levels of Aβ were significantly decreased (−24%, p = 0.04) in the CQ-treated animals (115 ± 8 ng/ml) compared to sham-treated controls (152 ± 18 ng/ml) (Figure 3E). There was a significant correlation (R^2 = 0.2, p = 0.03) between serum Aβ levels and total cerebral Aβ levels of the combined groups.

To determine the effects of treatment with CQ, we also measured metal levels (Al, Co, Cr, Cu, Fe, Mn, Pb, Se, Zn) in the soluble and sedimented fractions of brain

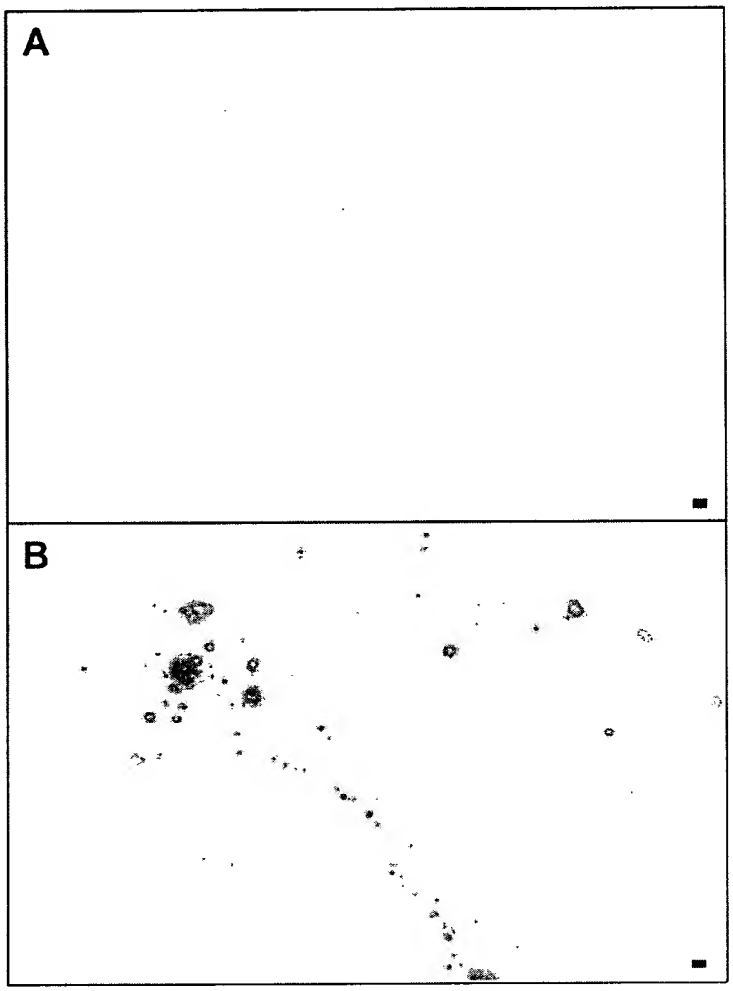

Figure 2. Initial Study of the Effects of Oral Treatment of 15-Month-Old APP2576 Transgenic Mice with Clioquinol

Immunohistochemistry of Aβ deposits in the hippocampal region of two 15-month-old APP2576 mice treated with either CQ 20 mg/kg/day (A) (representative of two animals) or sham-treated (B) (representative of three animals). The figure is typical of 4 sections analyzed throughout each brain. Size bar = 50 μm.

and peripheral organ homogenates from the 21-month-old APP2576 study cohort. We detected significant increases in Cu (+19%, +4.7 μM, $p = 0.007$) and Zn (+13%, +9.6 μM, $p = 0.006$) levels in the soluble cerebral fractions of the CQ-treated mice (Table 1), but no changes for any of the other metals measured. There was no significant change in any metal levels in the extracted centrifugation pellet fraction of the cerebral homogenates of the CQ-treated mice. There was a significant 24% increase in Co in the soluble fraction of the liver homogenates of the CQ-treated mice, as well as significant 15% increase in Zn content of the kidney pellet fraction. There were no other differences in metal levels in the liver and kidney samples from the CQ compared to the sham-treated animals. There were also no significant correlations between the levels or ratios of the various metals with the levels or ratios of the Aβ levels in the solubl and sedimented brain fractions, or with the plaque surface area. There was a significant linear correlation between Cu and Zn levels in the cerebral fractions of the combined (CQ + sham) cohort ($R^2 = 0.2$, $p = 0.03$).

As an appraisal of the potential toxicity of CQ, we reviewed the vital data of the two cohorts of 21-m nth-old APP2576 mice. Weight measurements of the sham-treated and CQ-treated mice taken at intervals throughout the study were not significantly different until day 53 of the study, when it observed that the mice treated with CQ maintained their weight, whereas the weights of the sham-treated animals declined so that the surviving CQ-treated mice became significantly heavier (37.41 ± 5.09 g) than the sham-treated mice (33.19 ± 4.05 g, $p < 0.05$) at day 53 (Figure 4A). CQ did not significantly affect the longevity of the mice, as the mean survival intervals of the CQ-treated mice (53.8 ± 4.3 d) and the sham-treated mice (57.2 ± 2.9 d) as well as survival curves (Figure 4B) were not significantly different (log rank survival distribution = 0.35, $p = 0.55$). Therefore, there was no gross evidence of toxicity for CQ treatment at this dose.

Because of the advanced age of these animals, water maze testing was not possible. However, to gauge gross physiological changes caused by CQ on the treated mice, we devised a 5 point integer scale that subjectively rated a combination of general features (motor activity, alertness, and general health signs) and was administered by a blind d operator v ry day to each individual mouse. There was a declin in the readings of the sham-treated mice that plateaued after 16 days of treatment (Figure 4C), which may have been due to repeated handling of the animals. In contrast, after the same initial decline as the control mice, the CQ-treated mice then

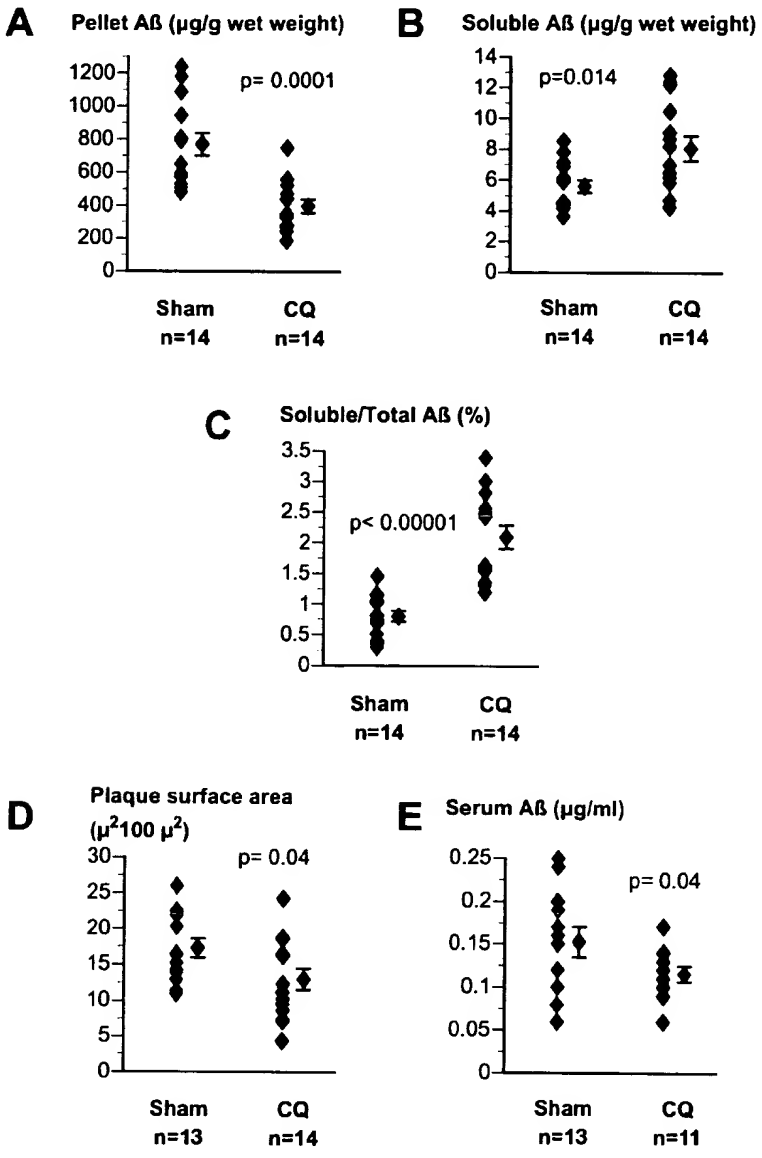

Figure 3. Effects of Oral Clioquinol Treatment on Aβ Metabolism in 21-Month-Old APP2576 Mice

(A and B) (A) Total and (B) soluble Aβ levels from cerebral homogenates of sham-treated mice and mice treated with clioquinol (30 mg/kg/day, CQ).

(C) Proportion of soluble Aβ compared to total Aβ (in percentage of mg/g wet weight values) in sham-treated compared to CQ-treated mice.

(D) Immunohistochemical plaque surface area from fixed cortical tissue of sham-treated mice and CQ-treated mice.

(E) Serum Aβ levels in sham-treated compared to CQ-treated mice.

recovered after 16 days, and their readings plateaued at a consistently higher mean score on this index than those of the sham-treated mice. This apparent benefit of CQ treatment was sustained from day 17 for each of the 46 remaining days of the study.

The plateauing in mean scores for each group follow-

Table 1. Effects of Oral Clioquinol Treatment on Distribution of Metals within Selected Tissues of 21-Month-Old APP2576 Mice

	Co (ng/g)		Cu		Zn	
	C	CQ	C	CQ	C	CQ
Brain (sol.)	18.1	12.3	1.70	**2.02****	4.83	**5.45****
Brain (pel.)	18.6	15.9	1.32	1.39	3.86	4.25
Liver (sol.)	27.1	**35.7***	3.26	3.41	29.6	34.0
Liver (pel.)	34.6	35.4	3.05	2.95	14.1	15.7
Kidney (sol.)	74.5	91.6	0.73	0.82	6.61	7.30
Kidney (pel.)	66.4	62.9	2.25	2.34	7.96	**9.12***

Metal levels (averages in ng or µg/g wet weight) in tissues where significant differences are noted between the C and CQ groups. Two-tailed t tests were performed on differences between the mean values from the sham-treated (C) compared to clioquinol-treated (CQ) samples. Significant differences in the mean values are represented by data in bold; asterisk, $p < 0.05$, and double asterisk, $p < 0.01$. No significant differences were found for levels of Al, Cr, Fe, Mn, Pb, or Se.

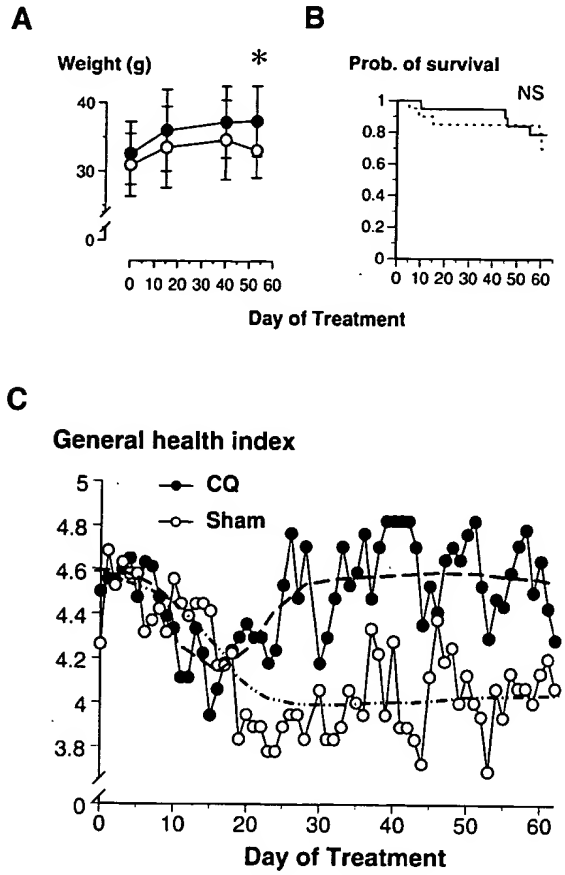

Figure 4. Effects of Clioquinol Treatment on the Systemic Health of 21-Month-Old APP2576 Mice

Parameters measured over the duration of the study: (A) weight (mean ± SD; asterisk, significant difference in mean weights in each group greater than the difference at day 0, $p < 0.05$; solid symbols are CQ treated); (B) survival curves (dashed is CQ treated); (C) general impairment (blinded subjective rating scale). Mean daily scores (±SEM) for the CQ-treated and sham-treated groups are indicated.

ing day 16 in Figure 3C is influenced by the integer scoring and nonlinear nature of the rating scale. A further breakdown of the daily scores (n = 746 individual observations) for CQ-treated group from day 17 onward revealed that 60.7% of the scores were 5 (out of 5, meaning no apparent impairment), 32.0% were 4 (meaning minor signs of impairment), 6.6% were 3 (meaning periodic signs of serious impairment), 0.7% were 2 (meaning persistent signs of serious impairment), 0.0% were 1 (meaning moribund). In contrast, the statistical mode for the sham-treated group was decreased to 4, with only 30.6% of daily readings being 5, 48.4% were 4, 12.6% were 3, 7.0% were 2, and 1.3% were 1 (n = 767 observations). Therefore, compared to sham-treated animals, treatment with CQ doubled the incidence of animals that appeared to be grossly normal (sham-treated = 30.6% of observations versus CQ-treated = 60.7%). Readings of 3 or less were relatively uncommon becaus onc the animals became serious impaired, they usually died soon after. A two-way ANOVA with repeated measures on these results indicated that the improvement in the surviving CQ-treated mic was significant [$F(61, 1525) = 2.4$, $p < 0.0001$; observati ns included from day 0 until completi n]. Alth ugh this scale is not a linear gauge of det rioration, the differences between the integer scores in the treated and untreated groups support the conclusion that CQ induces a conspicuous improvement in the health of the transgenic mice.

Discussion

Taken together, our findings indicate that CQ treatment, for as little as 9 weeks, inhibits and possibly reverses accumulation of Aβ deposits in APP2576 transgenic animals. Our in vitro findings that CQ reverses Cu^{2+}- and Zn^{2+}-induced Aβ aggregates (Figure 1A) and, at concentrations as low as 400 nM, solubilizes Aβ deposits in AD-affected postmortem brain tissue (Figure 1C) support the likelihood that the in vivo effect we observed was due to interdiction of the interaction of these metal ions with cerebral Aβ. This likelihood is further supported by the observation that CQ complexes with Zn^{2+} in the brain (Shiraki, 1979), especially in areas enriched in synaptic vesicular zinc such as the temporal lobe, which is severely affected by amyloid deposition. The alternative possibility of CQ acting as a fibril chain-breaker was not supported by NMR spectroscopy (Figure 1B).

The possibility that CQ exerted its effects by chelating $Fe^{2+/3+}$ (Kidani et al., 1974) cannot be excluded, since $Fe^{2+/3+}$ precipitates Aβ (less effectively than Zn^{2+} or Cu^{2+}) (Atwood et al., 1998) and is also found to be enriched in plaque (Lovell et al., 1998). But unlike the Zn and Cu levels in the brain, treatment with CQ did not alter Fe levels in the APP2576 cohort. Also, chelators that solubilize Aβ from postmortem AD cortical specimens appear to redistribute Zn and Cu, but not Fe (Cherny et al., 1999). It is unlikely that the decrease in Aβ accumulation was due to decreased Aβ synthesis caused by CQ-associated toxicity, since there was no decrease in brain APP levels, brain synaptophysin levels were not decreased, and the CQ-treated mice exhibited signs of improved general health rather than signs of toxicity (Figure 4C).

TETA did not inhibit Aβ deposition in this animal model. This may be because, unlike CQ, it is not a lipophilic molecule, and therefore may not be able to penetrate the Aβ deposits, or because the dose was insufficient. We have observed, however, that higher doses of TETA (40 mg/kg/d) were rapidly toxic in non-Tg mice, therefore limiting its testing. In contrast, CQ is rapidly absorbed from the rodent gut with blood levels reaching 1–10 μM within 1 hr of ingestion (Kotaki et al., 1983), and since it is hydrophobic, it passes rapidly into the brain. CQ is rapidly excreted in the urine so that a bolus dose of clioquinol is almost complet ly removed from the brain within 3 hr (Toyokura et al., 1975).

Our results indicate that the beneficial effects of CQ treatment contrast favorably with the popular Aβ vaccination experimental treatment approach and with any of the other reported candidate AD treatm nts tested in adult transgenic mice. Schenk et al. (1999) reported that total cerebral Aβ (including Aβ1-40 and Aβ1-42) in

PDAPP mice was decreased by 7.0 μg/g wet weight (at 15 months f age) after 4 m nths of monthly inoculation with synthetic Aβ1-42 compared to sham-treated contr ls, and by 13.8 μg/g (18 months of age) aft r 7 m nths of monthly treatment. Monthly treatment with anti-Aβ antibody injections for 6 months induced an 8.9 μg/g reduction in total cerebral Aβ1-42 in the same mouse model at 15 months of age (Bard et al., 2000). Since Aβ1-42 represents ~90% of total Aβ species in this model (Johnson-Wood et al., 1997), the estimated decrease in total Aβ reported by Bard et al. (2000) is ~10 μg/g. Recently, intranasal Aβ immunotherapy has been reported to induce a 1.5 μg/g decrease in Aβ in PDAPP transgenic mice (Weiner et al., 2000), and a replication of the original Aβ immunization protocol (Schenk et al., 1999) failed to decrease total Aβ but did induce a marked decrease in plaque surface area in TgCRND8 transgenic mice (Janus et al., 2000).

In comparison, we found a 375 μg/g reduction in extracted total cerebral Aβ with CQ treatment in 23-month-old APP2576 mice. Although this ~50% decrease compared to sham-treated controls is proportionally less than the best reported effects of the Aβ vaccination in older mice (60% and 80% decreases in 15- and 18-month-old PDAPP mice, respectively) (Schenk et al., 1999), the absolute reduction in Aβ induced by CQ is ~30 times greater. Furthermore, the beneficial effect of CQ was achieved more rapidly with CQ (9 weeks) than with the vaccine protocol (4 and 7 months).

Lim et al. (2000) recently reported a decrease in total Aβ of 22 ng/cerebral hemisphere (untreated controls = 54.7 ng, ibuprofen treated = 32.7 ng, approximately −40%) in the 2% SDS insoluble pellet fraction of 16-month-old APP2576 mice treated for 6 months with ibuprofen 56 mg/kg/d, an antiinflammatory drug. Contrast of our results with this study is difficult since we measured Aβ in PBS soluble and insoluble fractions; however, the PBS-insoluble values for our sham-treated 23-month-old APP2576 mice were 206 ± 25 ng/hemisphere, and for CQ treated 110 ± 11 ng/hemisphere, a difference of 96 ng/hemisphere or approximately −47% ($p = 0.002$), achieved following 9 weeks of treatment. The phosphatidylinositol kinase inhibitor wortmannin has been reported to prevent Aβ accumulation (0.2 μg/g) in the APP2576 mouse model treated for 4.5 months until 8.5 months of age (Haugabook et al., 2001). No data is yet available on the effects of wortmannin on APP2576 mice of similar ages to the groups that we studied.

CQ treatment was associated with absent histological amyloid deposition in two of five 15-month-old Tg mice (Figure 2). In our experience in examining Aβ immunohistochemistry in the brains of this APP2576 model, there has been no instance where amyloid deposition is absent at 15 months of age ($n > 200$ observations). Further studies will be necessary to determine whether CQ treatment prolonged for greater than the 9 and 12 week intervals employed here might induce mor instances of complete clearing of amyloid, such as those seen in the treatment of amyloid-bearing transgenic mice inoculated for 4–7 months with synthetic Aβ1-42 (Schenk t al., 1999). In relativ terms, treatment with Aβ immunotherapy or ibuprofen achieved greater proportional decreases in the surface area of Aβ immunoreactivity than th effects of CQ treatm nt (−25%, Figur 3D). However, direct comparison between rep rts that us quantitative image capture analysis of plaque surfac area is problematic for sev ral reasons. Th r ports of Aβ burden in units of surface area do not make reference to quantitative standards that correspond to a degree of staining intensity within a dynamic range. Therefore, the surfac area of the section that is adjudged as positive for Aβ immunoreactivity is a product of where the softwar is instructed to set the monochromatic threshold valu for a particular series of measurements. This threshold value varies from report to report, usually depending upon the nonspecific background intensity of the preparation, and the set zero is therefore arbitrary. As a result, separate studies of cortical Aβ burden in the sam mouse model result in greatly different values. For xample, Schenk et al. (1999) reported the mean area of cortical 3D6 Aβ immunoreactivity in sham-treated 18-month-old PDAPP mice to be 4.87%, yet the same group using the same methods subsequently reported that sham-treated younger (15-month-old) PDAPP mice hav a mean cortical 3D6 Aβ immunoreactivity that is apparently much greater (19%) than the older animals (Bard et al., 2000).

Furthermore, if the monochromatic threshold is set too high, many of the samples in a treatment group will achieve readouts that are below the threshold of detection, introducing artifactually decreased variance into the data from that group (because subzero values will be read as zero, with no variance). To address this problem, we set our threshold deliberately lower so that we could appreciate the variance in the treatment (CQ) group, which may explain why we observed a ~25% decrease in plaque surface area but the decreas in extracted Aβ was nearly twice as large (Figure 3, 50%). As a result of the arbitrary setting of threshold floor values for histological Aβ immunoreactivity, the proportional changes in plaque surface area values may not correspond to the changes in extracted Aβ levels. Without a means of standardizing such data, this methodological problem invalidates proportional comparis ns of plaque surface area with extracted Aβ values and also disqualifies the reference of plaque surface area changes in treated brains as a proportion of such surface area in untreated brains. Hence, we believ that the commonly used description of proportional changes in immunohistochemical plaque surface area within or between studies is an incorrect practice. Therefore, we have reported absolute changes in plaque surface area; the reduction in immunoreactive plaque surface area that we observed (4.3 μ^2/100 μ^2) was approximately the same as that reported by Schenk et al. (1999) (4.8 μ^2/100 μ^2), but additional controls would be necessary to validate such a comparison.

These methodological issues may contribute to th lack of correlation between extracted brain Aβ values and plaque surface area in human studies (McL an t al., 1999) and in the current study. However, plaqu may b a qualitative feature of Aβ accumulation produced by local neurochemical interactions, and not a strict product f Aβ concentration. This would explain why levels of Aβ are elevat d to the same extent in both neocortex and basal ganglia in AD (McL an et al., 1999), yet distinct plaques do not app ar in the basal ganglia.

Th biochemical distinction betw en plaque and nonplaque Aβ values could be important in valuating experimental treatment approaches in transgenic mouse models. For example, Aβ vaccination has recently been reported to decrease plaque surface area but not levels of total extracted Aβ (Janus et al., 2000). In contrast, the inability of CQ to resolubilize synthetic β-sheet-mediated Aβ aggregates (Figure 1A) may be compatible with CQ impacting less on plaque deposition than on diffuse Aβ deposition (Figure 3). Therefore, the two proposed therapies may be targeting different biochemical forms of Aβ. Both Janus et al. (2000) and our current study report in vivo improvements in the treated animals so that the relationship between physiological deficits and the accumulation of a specific species of Aβ is likely to be complex.

Given the caveats in making comparisons between these studies (e.g., differences in transgenic mouse models, differences in ages of cohorts, exponential accumulation of Aβ as the animals age, differences in extraction and assay procedures), it is not yet possible to draw firm conclusions about the relative potencies of CQ compared to other candidate treatment approaches. Further side-by-side comparative studies are required to achieve a true appraisal of the relative efficacies of these various treatment approaches. Nevertheless, the 375 μg/g reduction in total cerebral Aβ that was achieved with CQ is also meaningful because the concentration of total Aβ (using similar assay methods) in AD-affected neocortex is only 20–30 μg/g wet weight (Cherny et al., 1999; McLean et al., 1999). We therefore conclude from our results that CQ treatment at this dose leads to a marked interruption in cerebral Aβ accumulation that could potentially impact upon Aβ accumulation in AD, and that the dose of CQ per kilogram used in this study might exceed what would be needed for a beneficial effect in human clinical trials.

CQ treatment of the 21-month-old APP2576 mice elevated the concentration of soluble brain Aβ by 3.45 μg/g wet weight (Figure 3B). Although this is a ~50% increase in soluble Aβ levels compared to untreated animals, it represents only a ~1% rise in total Aβ levels (Figure 3C) and is overshadowed by the more profound (100-fold) decrease in insoluble Aβ (−375 μg/g, Figure 3A) leading to a net ~50% decrease in total Aβ burden. There is some concern that elevating soluble Aβ levels may contribute to pathophysiology since we (McLean et al., 1999) and others (Lue et al., 1999; Wang et al., 1999) have reported that the levels of soluble Aβ in AD cerebral tissue correlate with neuritic change, neurofibrillary tangle load, and inversely correlate with life expectancy, suggesting that soluble forms of Aβ may mediate toxicity in AD. Furthermore, toxic soluble forms of Aβ have been purified from AD-affected brain (Kuo et al., 1996). However, there was no evidence in the current study that the increase in soluble Aβ was accompanied by any adverse ffects, abbreviated lif span, or synaptic loss. Nontoxic soluble Aβ species are f und in normal brain tissue (Cherny et al., 1999; McL an et al., 1999). Also, not all forms of Aβ are toxic even in the AD-aff cted brain since there is a zinc-bound form whose abundance is inversely correlated with oxidative damage to neuropil (Cuajungco t al., 2000). It may be this form that is liberated into the soluble phase upon CQ treatment, such as when p stmortem AD-aff cted brain tissu is treated with chelators (Cherny et al., 1999) lik CQ (Figur 1B). Therefore, th soluble Aβ sp cies that is levated following CQ treatment is either a nontoxic form of Aβ or its toxicity has been attenuated by reaction with CQ.

The increase in the ratio of soluble to total Aβ could also represent a physiological normalization since, in AD, this ratio falls (Cherny et al., 1999; McLean et al., 1999), probably due to the reaction that drives the precipitation of the peptide in the disease. The CQ-associated rise in soluble Aβ levels associated with a net decrease in total Aβ implies that the Aβ deposits are dissociating into the soluble phase. The detection of elevated soluble Aβ levels in the CQ-treated mice also suggests that APP processing is not inhibited by CQ treatment. Therefore, in contrast to APP secretase inhibitors that decrease soluble Aβ levels, the CQ treatment is expected to increase soluble Aβ levels in the process of reversing Aβ deposition. Further studies will be necessary to determine whether cerebral soluble Aβ levels will rise further or ultimately fall if Aβ deposition was abolished by CQ, say as a result of more prolonged treatment of the mice.

Although treatment with a chelating agent may be expected to deplete systemic metal levels causing adverse effects, we found no depletion of peripheral metal levels. This result is probably a reflection of the low affinity of CQ for Zn^{2+} (K_1 = 7.0) and Cu^{2+} (K_1 = 8.9) so that once these metal ions are released from the cerebral amyloid mass, the affinity of the drug is too low to lead to net metal excretion in the face of the homeostatic response to maintain systemic metal levels. This result suggests that the drug may induce remission of amyloid deposition in AD itself without necessarily depleting tissue metal levels.

The 15% elevation in soluble Zn (+9.6 μM) and Cu (+4.7 μM) levels in the brain after treatment with CQ (Table 1) is surprising since CQ treatment of nontransgenic mice (10 mg/kg/d for 20 days) significantly decreased (−25%) brain Cu and Fe levels, although it did not change Zn levels (Yassin et al., 2000). We hypothesize that the rise in cerebral Zn and Cu levels in CQ-treated APP2576 mice may reflect the tissue scavenging of Aβ coprecipitated with Cu^{2+} and Zn^{2+} that is liberated by the action of CQ; the Aβ is proteolytically degraded while the metals are stored transiently in the metallothionein pool. It is also possible that CQ treatment has adjusted metal homeostasis in the tissue by altering the turnover of Aβ and APP. Studies of APP knockout mice indicate that Aβ and/or APP could be involved in Cu, Zn, and Fe homeostasis in the cerebral cortex and peripheral tissue, as evidenced by significantly increased Cu levels, and a trend toward increased Zn and Fe levels, in these tissues (White et al., 1999). Therefore, APP2576 mice that overexpress APP may be expected to have constitutively decreased levels of these metals. If Aβ plays such a role in metal homeostasis, CQ may indirectly correct the depletion of metals in th transgenic tissue by facilitating Aβ turnover. In agreement with this interpretation of the findings that clioquinol increases copper levels in APP2576 transgenic mice by correcting a defect in copper homeostasis, w have rec ntly found that these mice, as well as transgenic mic xpressing

the carboxy-terminal 100 amino acids of APP, both develop significantly decreased copper levels in brain tissue as they age (C. Maynard, R.A.C., C.L.M., A.I.B., R. Cappai, and Q.X. Li, unpublished data). This implicates the turnover of Aβ itself in sustaining brain copper levels.

CQ-treated animals did significantly better than sham-treated controls in an index of general impairment and also maintenance of body weight (Figures 4A and 4C). Despite being a crude instrument, the impairment index that we used detected a highly significant improvement in CQ-treated versus sham-treated mice. Further studies with more precise cognitive tests in younger animals capable of maze tasks are needed to determine whether this effect is due to systemic health effects or specific cognitive effects. Since clearance of Aβ accumulation (by vaccination) is associated with improved performance on cognitive testing of AD transgenic mice, we predict that the clearance of Aβ by CQ will also be accompanied by gains in cognitive performance. Nevertheless, our findings that the general well being of the animals improved with treatment raise the concern that studies of candidate treatments in transgenic mice will need to control for general health effects when testing performance in maze tasks. Improved maze performance may be a reflection of improved general health or alertness, and not necessarily of improved selective memory function. The blinded index that we employed is a facile and inexpensive means of introducing such a control.

We have shown that treatment with a lipophilic Zn/Cu chelator that crosses the blood-brain barrier attenuates Aβ deposition in a mouse model for AD, encouraging us to translate this novel approach into the clinical setting. Our results may explain how desferrioxamine, a parenteral chelator with affinities for Cu^{2+}, Zn^{2+}, Fe^{3+}, and Al^{3+}, may have acted to inhibit the progression of AD in a clinical trial (Crapper McLachlan et al., 1991).

Our results suggest that CQ may be suitable for testing in clinical trials in AD patients. The recommended dose of CQ when it was prescribed as an antiamebic was 500 mg 3–4 times/day (20 mg/kg/d), which is a weight-normalized dosage level that achieved inhibition of Aβ deposition in our current studies. A caveat in the clinical use of CQ is that it has been associated with subacute myelo-optic neuropathy (SMON), an uncommon neurological syndrome largely confined to Japan (Tsubaki et al., 1971). A causal relationship between CQ and SMON was never proven (Clifford Rose and Gawel, 1984; Meade, 1975), but the relatively low benefit of the drug balanced against the postulated risk of such a serious side effect led to its worldwide withdrawal in the early 1970s. Our current findings suggest that a reexamination of this drug and its side effects may be warranted.

CQ was used extensively for 20 years before the first case of SMON was described, and before its retirement, the drug was used for 500 million patient days as an antibiotic with a very favorable safety profile. SMON resembles an accelerated form of subacute combined degeneration due to vitamin B12 deficiency, and administration of CQ to normal mice has been reported to deplete brain and serum levels of vitamin B12 (Yassin et al., 2000). Nevertheless, no clear relationship has been identified between CQ dose and the risk for SMON in humans. Six cases of encephalopathy (but not SMON) induc d by acut overdoses in xc ss f 7.5 g have be n rep rted (Baumgartner et al., 1979). Conv rsely, 25% of patients with SMON (in a sampl of 2465 from Japan) had never taken CQ (Nakae t al., 1973). In th 1960s, the per capita consumption of CQ was higher in several other countries than the consumption in Japan. However, until 1975 there were 10,000 cases of SMON in Japan, while there were only 220 cases identifi d in the rest of the world. Therefore, it is possible that local demographic factors prevalent in Japan at the tim may have predisposed this population to develop SMON. One possibility is that the Japanese were endemically B12 deficient as a consequence of their diet in the post-war years and that this was the predisposing factor t SMON. CQ was commonly used to treat gastrointestinal symptoms in an unregulated manner in Japan in that era, and SMON usually begins with symptoms of abdominal pain and diarrhea, therefore overdosing in a B12-deficient population may have exaggerated the incidence of SMON in Japan. In light of this possible explanation for the association of CQ with SMON, coadministration of vitamin B12 is part of the phase two clinical trial that is currently in progress (C.L.M. and A.I.B., unpublished data). A completed phase one clinical trial of CQ with B12 supplementation in AD patients revealed no adverse systemic, neurological, or cognitive effects (C.G. Gottfries and M.X., unpublished data). Our current findings indicate that CQ and its derivatives, or other hydrophobic chelators, merit further investigation for their therapeutic utility in the prevention and treatment of AD.

Experimental Procedures

Effects of Chelators on Metal-Induced Aβ Aggregation

Aβ1-40 (10 ng in 200 μl) aggregation was induced by incubation (30 min, RT) with $ZnCl_2$ (25 μM in TBS, pH 7.4), $CuCl_2$ (5 μM in TBS, pH 6.8), or acidic conditions (pH 5.5, MES buffered saline). Aggregates were transferred to a 0.2 μ nylon membrane by filtration using a 96-well ELIFA apparatus (Pierce). The aggregates were then washed (200 μl/well) with TBS alone, TBS containing 2 μM EDTA, or TBS with 2 μM CQ. The membrane was fixed, probed with the anti-Aβ monoclonal antibody 6E10 (Senetek), and developed for exposure to ECL film. Quantification of retained, aggregated Aβ was performed by densitometry, calibrated against known amounts of the peptide.

NMR Spectroscopy

Six hundred megahertz 1H NMR spectra were taken of 0.3–1 mM Aβ peptides in 50 mM phosphate buffer (pH 6.9) 100 mM NaCl, 10% ^2H_2O at 271°K, using a Bruker DRX-600 instrument. CQ was first dissolved in DMSO and then added at 800 μM to ensure that the solution was saturated with soluble CQ. The maximal soluble concentration of CQ prepared in this manner was determined to be ~100 μM, so an aqueous suspension was formed. NMR peaks due to soluble CQ were detectable despite the presence of the majority of the CQ being in suspension.

Mice Studies

All mice were housed according to standard animal care protocols, fed *ad libitum*, and maintained in a pathogen-free environment at the MGH Neuroscience Center. The transgenic status of all animals was confirmed by PCR of tail snips, using the 3′UTR of the hamster cosmid PrP vector as a hybridization probe, and for overexpression on the APP transgene by Western blot (22C11) of postmortem brain tissue. The colony was maintained by Charles River Laboratories by crossing female Tg (HuAPP695.SWE)2576 with B6SJLF1/J males (Jackson Labs). The animals were randomized for therapy trials, coded, and the operators and data analysts remained double blind

to which treatment (or placebo) they received, until the code was broken at the completion of data collection. The treated animals were delivered, the chelator dissolved in 0.05% carboxymethylcellulose (Sigma) by daily gavage, and the untreated controls were gavaged with the placebo vehicle alone. The mice did not receive B12 supplementation.

The choice of dose of CQ for these studies was based upon pilot studies and a review of the literature. Preparatory studies determined that 4-month-old nontransgenic mice tolerated CQ at 40 mg/kg/day for 7 days with no apparent adverse effects. An SMON-like syndrome can be induced in dogs by sustained doses of CQ (>150 mg/kg/day), but mice and other rodents are far less susceptible to this syndrome (Tateishi and Otsuki, 1975). To minimize the chance of neurological side effects in our studies, we chose doses of 30 mg/kg/d or less for periods no greater than 12 weeks.

Mouse Data Collection

In the study of 21-month-old APP2576 mice, the animals were examined daily by a blinded operator, and a measurement of each animal's general behavior in its cage was taken by observation based upon a subjective 5 point rating scale, where 5 is alert, grooming, normal withdrawal response upon handling, and no obvious motor abnormality; 4 is either distressed or lethargic, not grooming, lost withdrawal response upon handling, but no motor abnormality; 3 is periodic obvious motor abnormality (paresis, spinning, tremor, rigidity); 2 is persistent motor abnormality or cachexia; and 1 is moribund. The animals were also weighed at intervals. Equality of survival distributions was statistically tested by log-rank analysis. All statistical analyses used Systat 9.0 (SPSS, Inc.). At the completion of all studies, the animals were anesthetized, a blood sample obtained, cardiac-perfused with cold saline, and the brain and peripheral organs removed. The left cerebral hemisphere was fixed in 4% paraformaldehyde, and the right hemisphere (without cerebellum) and remaining tissues were weighed and snap frozen in liquid nitrogen.

Aβ, APP, Synaptophysin, and GFAP Analyses

Snap-frozen tissues were thawed and homogenized in PBS (pH 7.4, 2 ml) and centrifuged at 100,000 × g for 30 min. Aβ in the supernatants (soluble), the pellet, in an aliquot of homogenate (total), and in serum, was quantified by Western blot using WO2, an anti-Aβ monoclonal antibody that detects all forms of full-length Aβ, calibrated with known quantities of synthetic Aβ, as previously described (Cherny et al., 1999; McLean et al., 1999). APP was quantified by Western blot from the same samples using 22C11 (Boehringer), which detects both the transgene-expressed human APP as well as the endogenous mouse APP, and using recombinant APP standards. This antibody is directed to the amino terminus of APP (Hilbich et al., 1993) and cannot differentiate between soluble and full-length APP; therefore, soluble and full-length APP levels were respectively measured from the supernatant and SDS- extracted pellet fractions of the cerebral homogenates after ultracentrifugation. Synaptophysin levels were measured in protein-normalized samples of total brain homogenate by Western blot (monoclonal antibody SY38, Boehringer), and relative values are reported in arbitrary absorbence units (U/g protein) after computer-assisted densitometric analysis of the films and ascertainment that the signals were in linear dynamic range (described in Cherny et al., 1999).

Formic acid extraction of tissue, while efficient, is problematic since the procedure chemically modifies Aβ (formication) and also requires the samples to be laboriously neutralized before PAGE analysis. For the measurement of PBS-insoluble Aβ in the brain homogenates, it was determined that the extraction of Aβ from the brain tissue by 70% formic acid (FA) treatment was no more efficient that extraction by 8% SDS sample buffer alone. The pellets remaining following PBS extraction and ultracentrifugation were resuspended 1:1000 (w/v) in PBS and aliquots were dissolved by extensive boiling in 8% SDS sample buffer containing 10% mercaptoethanol prior to separation on PAGE. No immunoreactive Aβ remained following this procedure and this was confirmed by examining subsequent FA extracts by Western analysis. The adoption of such a solubilization protocol that avoids the use of FA was developed in light of publications that observed that sequential extractions of human AD brain in various water based buffers or 10% SDS yielded further solubilization of Aβ from the formic acid "insoluble" pellet fraction (Harigaya et al., 1995; Tamaoka et al., 1994).

Furthermore, the Roher laboratory recently published that the brain Aβ deposits in the APP23 transgenic mouse model for Alzheimer's disease can also be fully extracted with SDS and, unlike human brain Aβ deposits, do not require FA treatment to be liberated (Kuo et al., 2001). Our own methodological experiments confirmed a similar observation in the brains of APP2576 mice. We found that in mice with advanced amyloid pathology (18–20 months), extraction of the brain homogenate into 8% SDS was 100% efficient, leaving no FA-extractable Aβ. This suggests, in agreement with Kuo et al. (2001), that the Aβ deposits in the transgenic mouse model contain less oxidative modifications than the Aβ that comprises amyloid in the human pathology and hence is more readily extracted by detergents. Therefore, apart from the pilot study of chelation treatment, brain homogenate samples were extracted into SDS sample buffer for Western blot.

Histological sections of whole brain were prepared and the proportional surface area of amyloid plaques estimated by computer-assisted immunohistochemical quantification (using monoclonal antibody 1E8), as described previously (McLean et al., 1999). Sections of the hippocampus were also stained with a monoclonal antibody against glial fibrillary acidic protein (DAKO) and the number of cells in the pyramidal layer staining positively per high-powered field (hpf) was determined (n = 3 fields, n = 3 sections). The operator remained blind to the CQ treatment status of the tissue. Data from the treated and untreated animal groups were analyzed by two-tailed t test.

Metal Quantification

Aliquots were taken from the supernatant samples of the tissue homogenates and diluted in 1% HNO_3. The pellets were freeze-dried and digested in 300 μl HNO_3, followed by 300 μl of H_2O_2 at 70°C, and further diluted in 1% HNO_3 for analysis by inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS was performed using an Ultramass 700 (Varian, Vic., Australia) in peak-hopping mode with spacing at 0.100 AMU, 1 point per peak, 50 scans per replicate, 3 replicates per sample. Plasma flow was 15 L/min with auxiliary flow 1.5 L/min. RF power was 1.2 kW. Sample was introduced using a glass nebulizer at a flow of 0.88 L/min. The apparatus was calibrated using a 1% HNO_3 solution containing Cu and Zn at 5, 10, 50, and 100 ppb with Y89 the internal standard for all isotopes of Cu and Zn. Metal values are mean μg/g wet weight of the original tissue sample. Two-tailed t test assuming unequal variances was performed on differences between the mean values from the untreated (C) compared to clioquinol-treated (CQ) samples.

Acknowledgments

This work was supported by funds from Prana Biotechnology Ltd, the National Institute on Aging, the American Health Assistance Foundation, the Percy Baxter Charitable Trust, and the NH&MRC. We thank Karen Hsiao Ashe for the APP2576 mice, Margaret Smith for assistance with genotyping, Maarten van den Buuse and Andrew McKinnon for statistical advice, and Craig Ritchie and Stephen MacFarlane for helpful discussion. The following authors are minor equity holders in Prana Biotechnology Ltd: R.A.C., K.J.B., R.E.T., C.L.M., and A.I.B.

Received December 11, 2000; revised March 29, 2001.

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EXHIBIT 2

January 30, 2003

Metal complexation with iodochlorhydroxyquin (clioquinol) targeting Aβ amyloid deposition and toxicity in Alzheimer's disease: proof-of-concept and safety.

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Running Title: Clioquinol in Alzheimer's disease

Word count: 4009

ABSTRACT

Background : The dementia of Alzheimer's disease (AD) is believed to be caused by the toxic accumulation of Aβ amyloid, due in part to excess binding of copper and zinc, metal ions that are abundant in the regions most affected. To test this Aβ amyloid theory of AD, we have developed a clinical intervention using a compound which binds copper and zinc, promotes the dissolution of aggregated Aβ, abrogates the toxic (H_2O_2-producing) properties of Aβ, and inhibits Aβ accumulation within the brain of a transgenic mouse model of AD.

Methods: Here we describe a Phase 2 clinical trial for the proof-of-concept for treatment of moderately severe AD patients, where the primary outcomes are efficacy (as measured by the ADAS-cog) and safety. Biochemical indicators include plasma Aβ amyloid levels and plasma zinc and copper levels.

Results: Thirty-six subjects were randomized [18 placebo and 18 clioquinol (CQ)]. Per protocol analyses were conducted on 32 subjects. The effect of treatment was statistically significant in the more-severely affected group (baseline ADAS-cog ≥ 25), but not the less-severely affected group (ADAS-cog < 25). The effect in the more-severely affected group was due to a substantial worsening of ADAS-cog scores in those taking placebo compared to negligible deterioration for the CQ group. Amongst the less-severely affected subjects, only minor worsening (not statistically significant) in ADAS-cog scores occurred in both placebo and CQ groups. Plasma $A\beta_{42}$ declined in the CQ group and increased in the placebo group. Plasma Zn levels rose by up to 30 per cent in the CQ-treated group. The drug was well tolerated by participants.

Interpretation: Subject to the usual caveats inherent in studies with small sample size, this phase 2 study has demonstrated proof-of-concept for a novel treatment strategy in AD. In subjects with

more-severe AD, there was little significant clinical progression after 36 weeks of treatment with CQ. Clinical efficacy was supported by biological changes underlying the etiology of $A\beta_{42}$ in Alzheimer's disease.

Key words: Alzheimer's disease, clinical trial, clioquinol, zinc, copper, Aβ amyloid.

There is now a general consensus on the theory that the causation of Alzheimer's disease (AD) lies within the pathway of the intracerebral biogenesis and accumulation of the Aβ amyloid protein (see recent reviews [1-2]). Although strongly supported by genetic and experimental evidence, the most convincing test of this theory will be the demonstration that drugs which target this pathway are efficacious in modifying the disease itself. Unravelling the proteolytic processing of the amyloid precursor protein (APP) which generates the Aβ amyloid has presented a number of therapeutic targets[3-5], and several of these approaches are at an early stage of clinical development. One such approach, immunization with Aβ to promote its clearance from the brain, has proven difficult with serious adverse effects[7].

We have developed a novel strategy of targeting the solubility and neurotoxicity of Aβ through disruption of Aβ-metal ion interactions. When Zn^{++} and Cu^{++} interact with Aβ, aggregation of Aβ into fibrils and plaques occurs[7-9]. At the same time, redox-active Cu^{++} -Aβ interactions can generate H_2O_2 from O_2 (ref 10). Both Cu^{++} and Zn^{++} can affect Aβ-lipid membrane interactions[11], a likely site for the toxic gain-of-function of Aβ. Compounds targeted to preventing Aβ-metal ion interactions should therefore have dual effects: promote solubilization of Aβ (and hence its clearance from the brain), and an abrogation of the Aβ-metal mediated toxic gain-of-function. One such lead compound, iodochlorhydroxyquin [PBT-1; an anti-infective agent also known as clioquinol (CQ)], a bioavailable Cu/Zn chelator, has been shown to promote the solubilization of AD plaque amyloid, to decrease the H_2O_2-generating capacity of Aβ, and cause a 49% decrease in brain Aβ deposition in a transgenic mouse model of AD[12].

CQ was withdrawn for oral use in 1970 due to its association with a rare neurological syndrome, subacute myelo-optic neuropathy (SMON), largely confined to Japan in the 1960s[13]. Recent

evidence suggests that SMON was caused by an overuse-related vitamin B_{12} deficiency [14] in an exceptionally vulnerable population, and therefore could be rehabilitated for study in a clinical setting[15]. A recent pilot study of CQ on AD patients treated with 20 mg and 80 mg/day for 20 days did not show any drug-related adverse effects[16].

On the basis of our preclinical data[12], we prepared a Phase 2 clinical trial of CQ for the treatment of AD. This Phase 2 study involved community dwelling patients with dementia. Because the primary outcome was efficacy, a double-blind design was chosen. A dose escalation schedule was chosen that would maximize the chance of detecting a cognitive change, whilst minimizing the risk of adverse effects. The starting dose of 3.3 mg/kg/day was within the same order of magnitude of the effective dose in the transgenic mouse model, but only about one tenth of the anti-infective dose.

In this manuscript, we report on the results demonstrating the possible disease-modifying effects of CQ (as measured by cognitive parameters and blood levels of Aβ) which provide the first human proof-of-concept evidence for the Aβ amyloid theory of AD.

METHODS

Ethical issues: In compliance with Australian laws concerning consent from individuals whose cognitive function may be impaired to the extent of being unable to make informed judgements or decisions, "Consent to Special Procedures" administered by the Victorian Civil and Administrative Tribunal was obtained for each participant not able to consent on their own behalf. In addition, third party consent was obtained from all carers. All subjects were stabilized

on donepezil prior to commencement of the study. The study was approved by the Royal Melbourne Hospital Research Foundation's Clinical Research and Ethics Committee.

Study population: The study took place at the AD clinical trials unit, Mental Health Research Institute of Victoria and at the Royal Melbourne Hospital. Criteria for inclusion in the study were: informed consent; a diagnosis of probable Alzheimer's disease by NINCDS-ADRDA criteria[17]; Alzheimer's Disease Assessment Scale-cognitive (ADAS-cog)[18] score of 20-45; Mini Mental State Examination (MMSE)[19] score of 10-24; on donepezil 5mg or 10mg for at least 6 months; relative or carer willing and able to support the trial; able to complete trial examinations; primary sensorial functions intact.

Patients were excluded if they had a history or clinical evidence of peripheral or optic neuropathy or had co-existing illnesses or past history that may have affected cognitive function, nerve conduction or illnesses that may have confounded the adverse event profile.

The following factors were obtained at baseline to determine if they correlated with outcome measures: age, sex, premorbid IQ [estimated from the National Adult Reading Test (NART)], years of education, and apolipoprotein E (ApoE) allotype .

Study design: The study was a double blind, placebo-controlled, parallel group randomized design. Thirty-six patients and their carers were recruited to participate, with patients randomized at a 1:1 ratio to receive either CQ or placebo. The duration of the study was 36 weeks. CQ oral dosage was 125mg bid from weeks 0-12, increased to 250mg bid from weeks 13-24, and finally, 375mg bid from weeks 25-36.

Study procedures: Screening procedures consisted of a full medical history, full physical, neurological and ophthalmic examination, blood and urine tests and psychometric tests (ADAS-cog, MMSE) to confirm the patient's eligibility for the study. Nerve conduction tests and visual evoked responses were conducted between the screening and baseline visits to provide a baseline measurement. Blood was collected for ApoE allotyping, baseline plasma levels of metals and Aβ prior to randomization. All patients continued their study entry dose of donepezil and all patients received 100 μg vitamin B_{12} intramuscularly every four weeks.

Outcome measures: The primary efficacy variable was a change from baseline score on the ADAS-cog conducted at baseline and at weeks 4, 12, 24 and 36. This measure was chosen to allow comparability of treatment effects with current therapeutics such as donepezil, where efficacy trials also used ADAS-cog as their primary outcome measure [20]. Although numerous neuropsychological tests could be considered as secondary measures, it was necessary to avoid fatiguing the subjects at review. Therefore the only other cognitive test was the Mini-Mental State Exam (MMSE). The CIBIC (clinician interview based impression of change incorporating caregiver information), a subjective observational index was also conducted. Plasma Aβ, and plasma zinc and copper were all taken 4 weekly.

Double antibody capture enzyme-linked immunosorbent assay (ELISA) for Aβ detection: Polystyrene plates were coated with mAb G210 (for Aβ40) or mAb G211 (for Aβ42). Plates were washed and biotinylated mAb WO2 was added. Bound antibody was detected with streptavidin-labelled Europium (Perkin Elmer, Vic Australia). The values obtained from triplicated wells were calculated based on standard curves generated on each plate.

Therapeutic drug monitoring: At weeks 12, 24 and 36, CQ blood levels were assayed by HPLC (Centre for Pharmaceutical Research, University of South Australia).

Safety measures: Standard adverse event reporting was conducted and biochemical tests, renal and liver function, full blood examination, serum vitamin B_{12} and folate levels were documented at each visit. To assess for peripheral and optic neuropathy a neurological examination was conducted at each visit, and visual evoked responses, nerve conduction studies and a full ophthalmic examination were conducted at screening, week 16 and prior to the final trial visit. An ECG was done at screening and weeks 12, 24 and 36.

Extension study: All patients who completed the double blind trial were invited to continue on a 48 week, prospective, open-label study of CQ which is still ongoing.

Data preparation and statistical analysis: Data monitoring and management were undertaken by independent contractors (Kendle International and Health Research Solutions, Melbourne). Evidence for efficacy was indicated by a significant difference in change from baseline between treatment arms. Analysis of variance was the principal method of evaluating statistical significance with the treatment arm illness severity at baseline being the primary design factor. Potentially significant covariates were introduced as necessary. Differences between groups on categorical measures were analysed using exact statistical methods in order to maximise power. Based on the assumption of a correlation of 0.60 between measurement occasions, power to detect an effect of one standard deviation difference in change between groups from baseline to week 36 would have been approximately 80% if 15 subjects were recruited per group. Since an attrition rate of 15% has been observed in similar populations, 18 patients were recruited into each arm.

The baseline illness severity factor was created, as planned, by division of the sample into two groups at the median ADAS-cog score at baseline, yielding less-severely and more-severely affected groups (n=8 and 8 in the treatment arm and n=7 and 9 in the placebo arm, respectively).

Role of funding sources: The basic research and assays required for this study were developed in part from support from the NH&MRC and the Baxter Trust. The clinical trial itself was supported in part by Prana Biotechnology.

RESULTS:

Subject recruitment and demographics: Thirty six subjects were recruited over a 12 month period commencing April 2000 (Fig 1). Of these, 32 had sufficient data for per protocol analysis. Two subjects were lost from each arm. In the placebo arm, one subject died and another withdrew because of illness unrelated to AD. In the treatment arm, one subject withdrew because of behavioral changes associated with AD (paranoia, leading to non-cooperation with testing). Another subject was not included in the analysis because their initial diagnosis of AD was probably incorrect (the symptoms and signs evolved into a picture characteristic of Diffuse Lewy Body disease). Exclusion of this subject had no effect on the outcomes of this study. A third subject withdrew at week 24 due to unrelated medical problems. Data for this subject were included in all analyses except those involving week 36. Five subjects (three in treatment and two in placebo arms) did not tolerate the final dosage increase (375mg bid) and were returned to the previously tolerated dose (250mg bid) for the remainder of their involvement in the study.

The groups did not differ across all demographic, biological and clinical parameters at baseline (Table 1), other than the treatment arm having a higher mean premorbid IQ than the placebo group as estimated using the NART (111.4 compared to 104.9; $t(30)=2.27$, $p=0.031$) and a lower level of thyroid stimulating hormone (TSH) (1.14 compared to 2.00 mU/L; $t(30)=4.400$, $p<0.001$). The NART and TSH were subsequently provisionally entered into analyses as co-variates but were found to be not significant in any analysis.

Proof-of-concept: Ideally, any drug targeting the Aβ amyloid pathway should have two principal effects: a disease-modifying effect as assessed by cognitive parameters and a biological response assessed by measurement of Aβ in blood, CSF or the brain itself.

Changes in the ADAS-cog score at weeks 4, 12, 24 and 36 from baseline were subject to two-way analysis of variance with factors of treatment arm and baseline illness severity. As planned in the protocol, the effect of severity of illness was examined by stratification of the sample into subjects less- or more- severely affected than the median value of the base line ADAS-cog (values <25, ≥ 25). At baseline there were no significant or near-significant differences between the main effect of treatment arm [$F(1,28)=0.21$, $p=0.647$]. Similarly, there were no significant differences between treatment arms at either level of severity. The main effect of treatment arm was not significant at any week, although trends toward significance were noted at week 4 [$F(1,28)=3.55$, $p=0.070$] and week 24 [$(F(1,28)=3.31$, $p=0.080$] (Fig 2A). Simple effects tests within level of severity showed the main effect to be separable into non-significant results for the less-severe stratum on all weeks and significant differences in the more-severe stratum at weeks 4 [$F(1,28)=7.73$, $p=0.010$] and week 24 [$F(1,28)=6.63$, $p=0.016$] (Fig2B). This trend was maintained at week 36 but marginally escaped statistical significance [$F(1,28)=3.62$, $p=0.068$]. The difference in mean change from baseline ADAS-cog score of CQ over placebo at weeks 24

and 36 was a difference of 6.36 (95% CI: -0.50 – 13.23) and 7.37 (95% CI: 1.51 – 13.24) respectively.

The MMSE, a less sensitive measure of cognitive impairment, showed a similar pattern without reaching significance. By contrast, ADAS – noncog and CIBIC did not show any clear differences or trends. ApoE genotyping did not disclose any effect other than an over-representation of the ε4 allotype in both groups.

There were no significant differences in baseline plasma $A\beta_{42}$ levels between treatment arms or severity strata. Plasma $A\beta_{42}$ showed a significant decline from baseline in the CQ-treated group from week 20 onwards; over the same time, plasma $A\beta_{42}$ in the placebo group increased (Fig 3A). Stratification by illness severity as above demonstrated that changes were evident only in the less-severely affected (Fig 3B). The wide variance in individual levels at baseline in plasma $A\beta_{40/42}$ led to reduced power of the study to detect any significant differences in mean changes between groups. The relative stability of individual $A\beta_{42}$ values therefore emerges as a potent window on cerebral Aβ metabolism.

Analysis of plasma $A\beta_{40}$ levels showed overall similar trends, with significant differences between placebo and CQ groups observed at weeks 8, 32, and 36 in the less-severely affected groups. For individuals, there was a highly significant ($p< 0.0001$) correlation between $A\beta_{42}$ and $A\beta_{40}$ levels.

Effect on plasma Zn and Cu: Administration of CQ was associated with a significant elevation of total plasma Zn (Fig 4A) but with no effect on plasma Cu (Fig 4B). Samples collected with an

indwelling catheter at weeks 12, 24 and 36 were found to be unreliable for technical reasons and were therefore omitted from this analysis (the metal levels in blood collected by this technique were depressed, compared to samples taken on other visits by single vein puncture, probably as a result of platelet activation). Mean absolute levels of Zn (9.4 μM) in all groups at baseline were below age-related normative values[21]. Mean absolute levels of Cu (13.1 μM) were within the age-related normative range[22]. Correlation of plasma $A\beta_{42/40}$ levels with Zn/Cu levels assayed on the same or subsequent occasions showed no significant associations.

Blood levels of CQ: Steady state pre-dose levels of CQ at total daily dosages of 250, 500 and 750 mg were 4.03±2.10, 6.74±3.70, 7.60±2.15 μg/ml, respectively, and did not show significant correlations with ADAS-cog, metal levels or Aβ levels assayed on the same or subsequent occasions.

Safety results and analysis: Safety analysis was conducted on all data irrespective of the stage reached in the trial. There were a total of 111 attributable adverse events (AE) reported, 61 in the treatment group and 50 in the placebo arm. The mean number of discrete events per subject was not significantly different between arms (Table 2). Five subjects developed serious adverse events (SAE). A 66-year-old female with hypertension and hyperlipidemia developed impaired visual acuity and color vision after having completed the trial and receiving CQ 375mg bid. This was considered to be probably attributable to CQ and her symptoms rapidly resolved upon its cessation. Four non-attributable SAE were recorded. There was one death due to intracranial hemorrhage (placebo) and three hospitalizations: for hip pain (placebo), syncope due to impaired cardiac function (CQ) and confusion (placebo).

Cardiac safety: Symptoms of postural cardiac insufficiency were common with 27/36 (75%) of subjects reporting this on at least one occasion. There were no significant between-group differences (Table 2).

Neurological safety: The development of new or worsening neurological symptoms or signs were uncommon. Analyses of the nerve conduction data revealed that there were no relevant or significant between-group differences. There were also no significant differences in the rates of impairment or deterioration in visual acuity, color vision, visual fields or of fundoscopic abnormalities between the two groups (using McNemar's test for paired categorical data).

Gastrointestinal safety: Subjects on CQ experienced fewer occurrences of diarrhea but had some changes in liver function tests (LFT). In the CQ arm, within-group analyses of γ-GT, AST and ALT showed intermittent significant elevations from baseline at various time points which were normalising by week 36. The only significant between-group difference occurred with γ-GT. No subject developed any overt symptoms or signs of impaired liver function.

Hematological safety: There were no hematological AE noted in the CQ treated subjects. However, there was a significant reduction in hemoglobin in the CQ arm over the course of the study, which by week 36 was 9.6 g/L ($F(1,27) = 6.135$, $P = 0.02$). Between-group differences were also observed at weeks 24 and 36. Corresponding changes in packed cell volume and red cell count were observed. The cause of these clinically insignificant decreases was uncertain. For all other hematological parameters, there were no significant between-group differences.

Renal safety: Serum creatinine was mildly elevated in both arms over the course of the study and a small significant drop in serum albumin was noted in the CQ arm, however there were no

significant between-group differences in these parameters. Serum urea remained unchanged in the placebo arm, and increased slightly in the CQ arm at weeks 4 and 8 with between-group analysis showing significant differences at weeks 12 and 36.

DISCUSSION

The data are offered as a proof-of-concept that a low molecular weight compound (CQ, mass 306d) targeting the Aβ pathway can have a significant disease-modifying effect on the natural history of AD. As such, these data are the first of what will hopefully become a series of independent observations, using other therapeutic strategies, confirming the Aβ amyloid theory of AD. The benefit of CQ in this study population was only seen in the more-severely affected subjects, probably due to the low power of the study and the limited sensitivity of the ADAS-cog to detect subtle cognitive differences in the less-severely affected groups over the nine month study period. It is worth noting that the statistically significant separation of 3 ADAS-cog points achieved after 24 weeks treatment with the acetylcholinesterase inhibitor, donepezil (Aricept), required a study population of more than 300 subjects[20]. The present study with an order-of-magnitude smaller population size, underscores the potential impact of the 7 ADAS-cog points difference observed in our study at 24 weeks (Fig1) if replicated in a future, larger, trial of CQ. The significant benefit seen in the more-severely affected treatment group at 4 weeks is also of interest, as this may represent the short-term effect of CQ neutralizing the neurotoxicity of the soluble pool of Aβ (less than 1% of the total Aβ burden[23]), a pool which is also accessible through immunomodulatory interventions[24]. Such a rapid clinical effect would also be consistent with that reported for CQ in a pilot, 3 week, open-label study at comparatively low dosage[16].

Perhaps more convincing than the cognitive effects of CQ are the data which show a lowering of plasma $A\beta_{42}$. Previous cross-sectional assays of blood Aβ levels have been disappointing because of large inter-individual variations[25], although one longitudinal study of pre-clinical AD disclosed higher plasma $A\beta_{42}$ levels[26], and within-pedigree measurements of plasma $A\beta_{42}$ in late onset AD have indicated a strong genetic effect[27]. The present longitudinal study appears to be the first to have followed affected individuals at regular intervals over an extended period, disclosing a progressive increase in plasma $A\beta_{42}$, principally in the less-severely affected group. There was a trend over time towards lower mean levels in the more-severely affected group (data not shown), which is consistent with the large body of data on CSF Aβ levels which show an elevation early in the disease, followed by a progressive fall as the disease evolves[28]. A 24% decrease in serum Aβ was also observed in CQ –treated Tg2576 mice models of AD[12]. The most parsimonious explanation of these observations is that Aβ is being sequestered into cerebral extracellular spaces as the disease unfolds. The pool of Aβ in equilibrium with the CSF may be different from that in the blood. Both blood and CSF Aβ pools could have non-cerebral contributions (for example, from platelets in the blood[29] and the choroid plexus in the CSF[30]) yet it would appear from the present data that plasma Aβ may yet emerge as a reliable surrogate marker for cerebral Aβ. The unexpected rapid turnover and efflux into plasma of cerebral Aβ in experimental animals[31] would suggest that active clearance/ degradative mechanisms are operating.

>From current experimental data, we surmise that CQ may be having at least two principal therapeutic effects on Aβ: promoting its zinc-dependent solubilization and hence clearance/degradation from the brain and also diminishing the toxic gain-of-function mediated by Aβ-copper interactions. The 30% increase in plasma Zn (from a below-normative baseline level)

associated with CQ treatment might arise from an exchangeable pool, whereas the lack of effect on blood Cu levels might reflect a more rigidly controlled metabolic pool. Experimental studies of CQ on mouse brain Zn and Cu levels showed increases of 13% (Zn) and 19% (Cu) in the soluble fractions[12]. Given the uncertainty in the proposed mechanism of action of CQ, it is difficult to predict *a priori* what an effective therapeutic dose might be. Experimentally, a molar ratio of $[CQ]_{EC}$:$[Metal]_{free}$: $[A\beta]_{sol}$ of at least 4:2:1 (where $[CQ]_{EC}$ is the effective extracellular concentration of CQ in the brain, $[Metal]_{free}$ is the concentration of free metals such as zinc ions in the peri-synaptic spaces of the glutamatergically-innervated cerebral cortex and hippocampus, and $[A\beta]_{Sol}$ is the concentration of the soluble "toxic" species of Aβ in the same extracellular cerebral compartment) should be required for the short- and longer-term neutralization of H_2O_2 production. The measured basal levels of plasma CQ in the current study ranged from 13 to 25μM. Allowing for a large proportion of CQ being bound to serum protein (albumin) or lipoproteins[32], the available active compound in the brain should be approximately 100-200 nM. The concentration of total Aβ in the AD brain varies considerably, but is estimated to range from the low nM to low μM range[23, 33], of which less than 1% is available as a "toxic" soluble species[23]. Actual measurements of brain $[CQ]_{EC}$ will be required, together with plasma pharmacokinetics, before a more rational approach to dosing can be applied, but the current available data suggest that the dosages employed in this study may be within a theoretical optimum. Other small drugs targeting the systemic amyloid pathway have been shown to operate with an experimentally determined stoichiometry of 5:2(ref 34), but have been used in human trials at much higher doses (40mg/kg/day) than those employed in the present study.

Safety issues were always of paramount importance in a study involving the chronic administration of a drug with a known history of adverse events. We balanced the risk of treating a malignant disease such as AD against the relatively low risk of developing SMON by careful

regular evaluations, ensuring complete normality of vitamin B_{12} and folate metabolism, and a preparedness to withdraw subjects at the first sign of SMON. CQ had no overall effect on nerve conduction, either optic or peripheral. Optic neuropathy was suspected in one subject, whose visual symptoms resolved rapidly upon treatment cessation. Objective measures of optic nerve conduction demonstrated bilateral decreased amplitude in visual evoked potentials at week 24 with further decrease at week 36. CQ may have been associated with optic neuropathy in this case, though evidence of a direct causal link between CQ and optic neuropathy is uncertain based on the results from this study. Disturbances of color vision and other ophthalmologic changes are known to occur during the natural history of AD [36-38].

The association between CQ and transient elevation of transaminases at week 24 may represent enzyme induction, with subsequent regression. The asymptomatic decrease in hemoglobin, where all subjects remained within the normal range, without changes to any other hematological parameter may reflect hemolysis or depletion of iron stores. Mild changes in renal function are unlikely to reflect significant nephrotoxicity of this drug. Further studies will be required to elucidate the mechanisms underlying all of these biochemical changes.

At the time of writing, 27 subjects had agreed to participate in an open-label extension study of CQ. More than 10 subjects have now been on this drug at 750 mg per day for more than 9 months. None has developed any CQ-attributable adverse events (this cohort will be the subject of a future publication). We conclude that the safety profile and apparent efficacy of CQ in this population are sufficiently encouraging to allow future trials to take this investigation of a novel therapeutic intervention, targeting Aβ amyloid, to the next phase.

Author contributions:

Study concept and design: C Ritchie, A Mackinnon, M Mastwyk, M Xilinas, D Ames, S Davis, C Masters; Acquisition of data: S Macfarlane, M Mastwyk, L MacGregor, L Kiers, R Cherny, QX Li, A Tammer, D Carrington, C Mavros, I Volitakis; Analysis and interpretation of data: C Ritchie, A Mackinnon, S Macfarlane, M Mastwyk, R Cherny, QX Li; Drafting of manuscript: C Masters; Critical revision of manuscript for important intellectual input: C Ritchie, A Mackinnon, S Macfarlane, D Ames, S Davis, K Beyreuther, C Masters; Statistical expertise: A Mackinnon; Administrative, technical and material support: M Mastwyk, L MacGregor, R Cherny, A Tammer, D Carrington, C Mavros, I Volitakis; Study supervision: S Macfarlane, M Mastwyk, C Masters.

Conflict of Interest Disclosure: Dr Craig Ritchie has been paid for his time and effort in analyzing the results from the trial sponsor, Prana Biotechnology. Dr Steve Macfarlance, Ms Maree Mastwyk and Dr Lachlan MacGregor were employed by the Mental Health Research Institute on funds provided by Prana Biotechnology. Prof Colin Masters is a Director of Prana Biotechnology, Chair of its Scientific Advisory committee, and a minor stockholder. Dr Robert Cherny is a minor stockholder in Prana Biotechnology. Professor Konrad Beyreuther is on the Scientific Advisory Committee of Prana Biotechnology.

Acknowledgements: Funded in part by grants from the NH&MRC, Prana Biotechnology, and the Baxter Trust. The authors express their appreciation of the generosity of the volunteer subjects and their carers who participated in this study; also to the Safety Committee (A/Prof E Chiu, Drs S Collins and M Hopwood, D LoGiudice, J Merory), Ms R Shafiq-Antonacci for

performing the CIBIC, Drs K Barnham, D Darby, P Desmond, and K Taubman for advice and consulting, and Ms F Fraser for assistance.

Legends

Fig 1

Outline of the flow chart of subjects studied.

Fig 2

Mean change (±SE) over time from baseline in cognitive abilities (as assessed with ADAS-cog) in (A) two arms of CQ vs placebo and (B) stratification by severity within treatment arms [less-severely affected (ADAS-cog < 25), more-severely affected (ADAS-cog ≥25) (*$p \leq 0.05$; ** $p \leq 0.01$).

Fig 3

Mean change (±SE) over time from baseline in plasma $A\beta_{42}$ levels in (A) the arms of CQ vs placebo and (B) stratification by severity as in Fig 2. (***$p \leq 0.001$)

Fig 4

Mean change (±SE) over time from baseline in (A) plasma Zn (B) plasma Cu in the two arms of CQ vs placebo.

TABLE 1. Baseline demographics and key clinical variables

Variable	Total Sample (n=32)	Group: Clioquinol (n=16)	Group: Placebo (n=16)	P Value
Age mean (SD; min-max)	72.50 (8.37; 56-87)	73.19 (8.61; 58-87)	71.81 (8.35; 56-87)	P=0.65†
Sex (n; % male)	17 (53.1%)	8 (47.1%)	9 (52.9%)	P=1.00‡
ApoE status				
ApoE4 heterozygote n (%)	15 (46.9%)	7 (43.8%)	8 (50.0%)	
ApoE4 homozygote n (%)	3 (9.4%)	2 (12.5%)	1 (6.3%)	P=1.00‡
Estimated premorbid IQ NART mean, (SD; min-max)	108.1 (8.86; 91-124)	111.4 (8.04; 94-121)	104.9 (8.26; 91-124)	P=0.03†
ADAS-Cog	26.31 (7.27; 15-46)	25.56 (7.67; 15-46)	27.06 (7.01; 19-41)	P=0.57†
Age of first diagnosis mean, (SD; min-max)	70.09 (7.98; 54-83)	70.88 (8.50; 57-83)	69.31 (7.61; 54-83)	P=0.59†
Duration of illness (years) mean (SD; min-max)	2.41 (1.19; 1-5)	2.31 (1.08; 1-4)	2.56 (1.32; 1-5)	P=0.66†

† Independent sample t-test (all tests 30 *df*)
‡ Exact, two-tailed test.

TABLE 2. ATTRIBUTABLE ADVERSE EVENTS WITH A RISK OF GREATER THAN 10% IN EITHER ARM OR WHERE POINT ESTIMATE RISK RATIO IS GREATER THAN 2.0 OR LESS THAN 0.5

	Treatment (n=16)	Placebo (n=16)	Relative Risk (95% CI)
Cardiovascular			
Postural hypotension	12	11	1.09 (0.67-1.79)
Postural tachycardia	12	8	1.33 (0.74-2.40)
Postural dizziness	7	3	2.33 (0.71-7.63)
Subjects with ≥1 postural symptom	13	14	0.93 (0.64-1.36)
Neurological			
Impaired nerve conduction	3	1	3.0 (0.34-26.2)
Impaired reflexes	1	2	0.5 (0.05-5.04)
Numb legs	2	0	-
Subjects with ≥1 symptom	6	4	1.5 (0.51-4.43)
Gastrointestinal			
Diarrhea	1	4	0.25 (0.03-2.02)--2.0 (0.2-
Constipation	2	0	20.1)1.25 (0.4-3.91)
Nausea	2	0	
Abdominal pain	2	1	
Subjects with ≥ 1 symptom	5	4	
RenalMicroalbuminuria			
	5	5	1.00 (0.35-2.87)
HematologicalLymphopenia			
	0	3	-
Liver Function Tests Raised γGTRaised			
bilirubin**Subjects with ≥1 abnormal**	2	1	2.0 (0.2-20.1)-
	2	0	4.0 (0.49-32.4)
	4	1	
Other			
Decreased vitamin B_{12}	0	2	-
Mean number of discrete adverse			
events per subject (SD)	3.38 (2.14)	2.78 (1.48)	Mean diff 0.611
			(95% CI -0.64 - 1.89 p=0.327)

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Figure 1

Subject Accountability

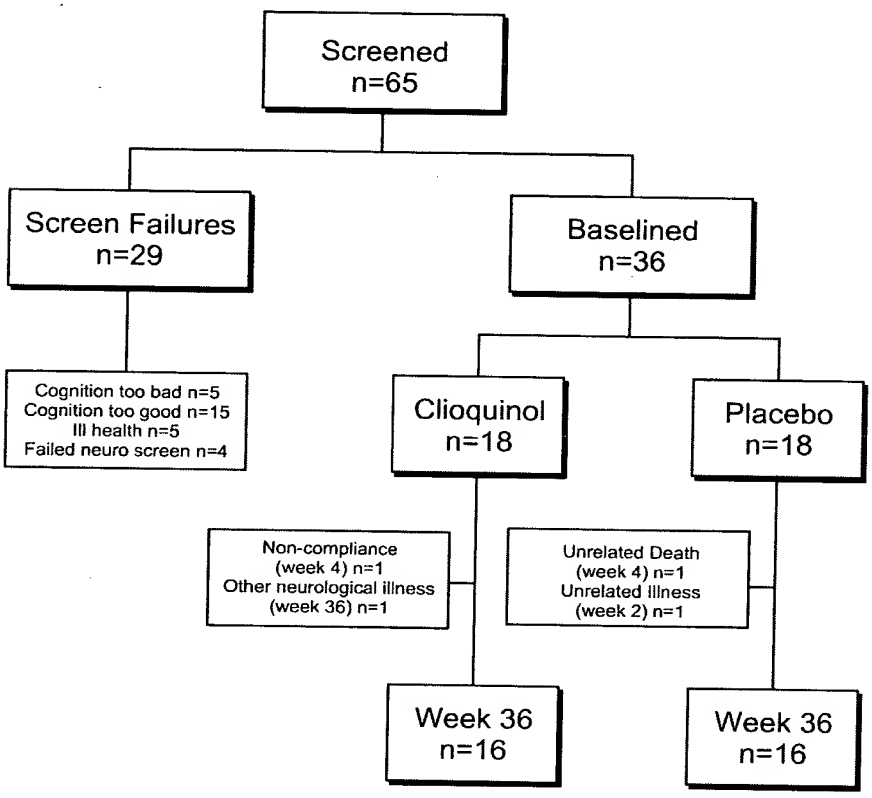


Figure 2

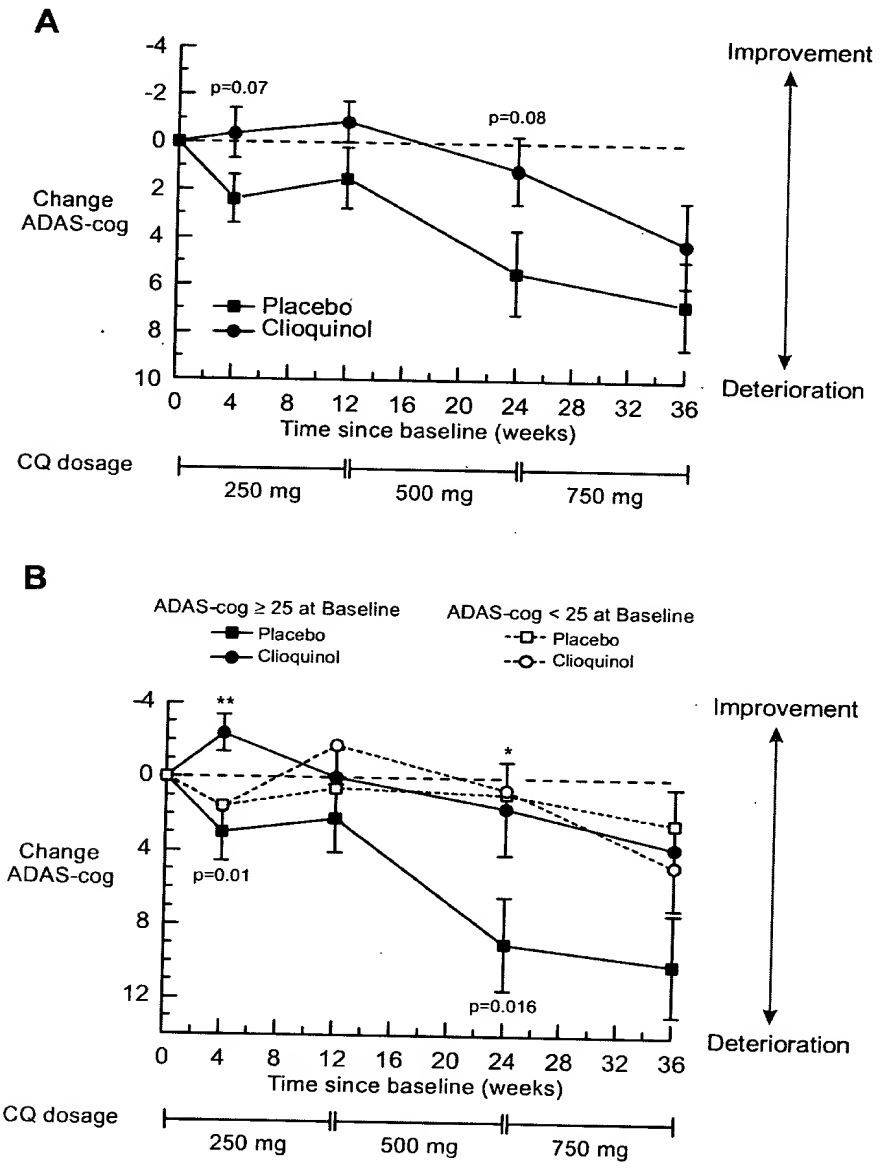

A
Improvement
Deterioration
Change ADAS-cog
p=0.07
p=0.08
Placebo
Clioquinol
Time since baseline (weeks)
CQ dosage
250 mg
500 mg
750 mg
B
ADAS-cog ≥ 25 at Baseline
Placebo
Clioquinol
ADAS-cog < 25 at Baseline
Placebo
Clioquinol
Improvement
Deterioration
Change ADAS-cog
**
*
p=0.01
p=0.016
Time since baseline (weeks)
CQ dosage
250 mg
500 mg
750 mg

Figure 3

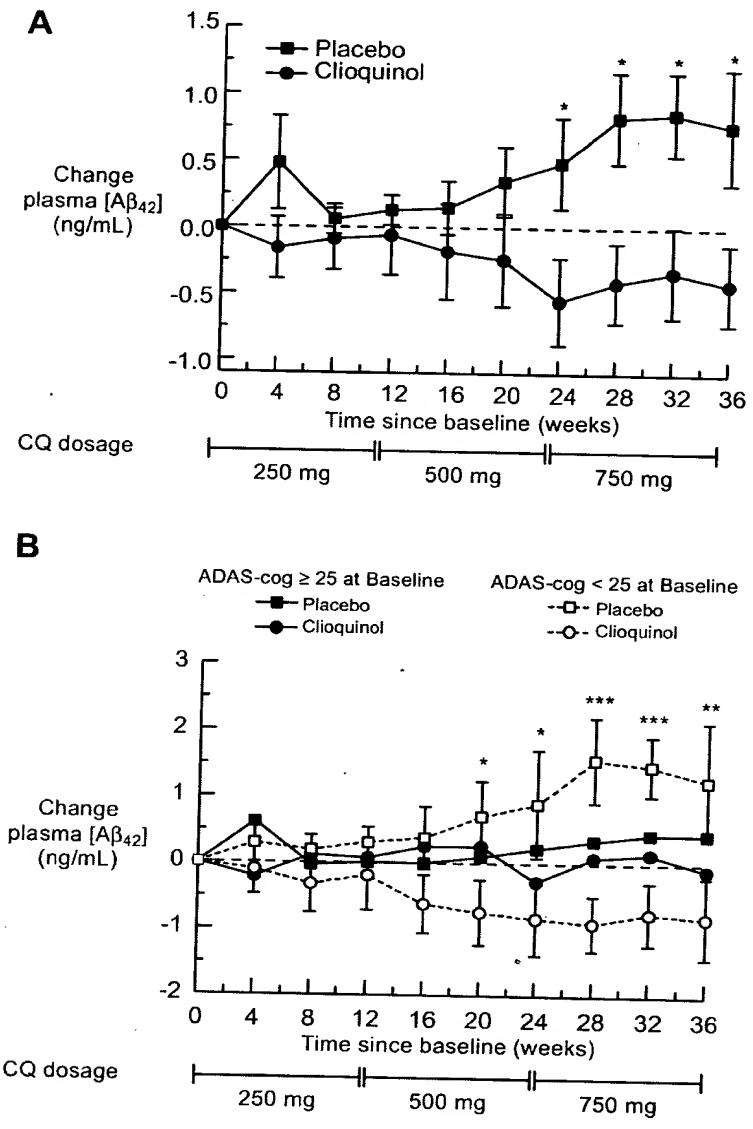
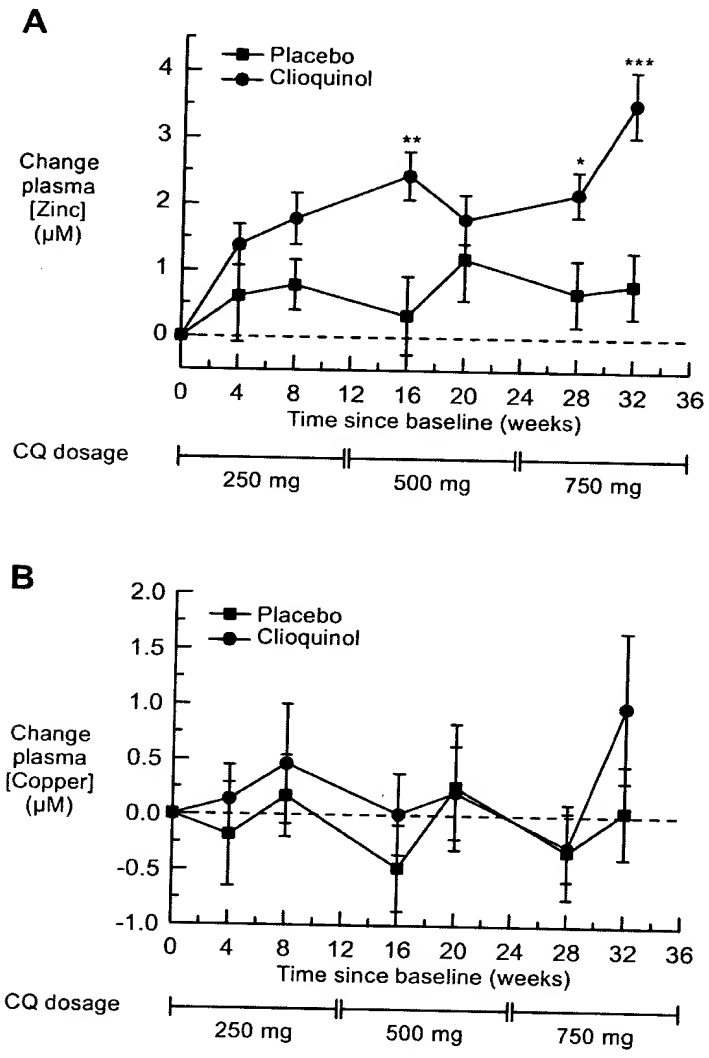

A
Placebo
Clioquinol
Change plasma [Aβ42] (ng/mL)
1.5
1.0
0.5
0.0
-0.5
-1.0
0 4 8 12 16 20 24 28 32 36
Time since baseline (weeks)
CQ dosage
250 mg
500 mg
750 mg
B
ADAS-cog ≥ 25 at Baseline
Placebo
Clioquinol
ADAS-cog < 25 at Baseline
Placebo
Clioquinol
Change plasma [Aβ42] (ng/mL)
3
2
1
0
-1
-2
0 4 8 12 16 20 24 28 32 36
Time since baseline (weeks)
CQ dosage
250 mg
500 mg
750 mg

Figure 4


A
Placebo
Clioquinol
Change plasma [Zinc] (μM)
4
3
2
1
0
**
*
***
0 4 8 12 16 20 24 28 32 36
Time since baseline (weeks)
CQ dosage
250 mg
500 mg
750 mg
B
Placebo
Clioquinol
Change plasma [Copper] (μM)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
0 4 8 12 16 20 24 28 32 36
Time since baseline (weeks)
CQ dosage
250 mg
500 mg
750 mg